THE INNOVATIVE TECHNOLOGY TO PRODUCE LIQUID AND SOLID INOCULANT OF *Bradyrhizobium diazoefficiens* USDA110

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

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นวัตกรรมเทคโนโลยีการผลิตหัวเชื้อ *Bradyrhizobium diazoefficiens* USDA110 ในรูปแบบชนิดเหลว และชนิดผสมในวัสดุพาหะ

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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บิดู อภัยโส : นวัตกรรมเทคโนโลยีการผลิตหัวเชื้อ *Bradyrhizobium diazoefficiens* USDA110 ในรูปแบบชนิดเหลว และชนิดผสมในวัสดุพาหะ (THE INNOVATIVE TECHNOLOGY TO PRODUCE LIQUID AND SOLID INOCULANT OF *Bradyrhizobium diazoefficiens* USDA110) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร.หนึ่ง เตียอำรุง, 103 หน้า.

แบรดี้ไรโซเบียมเป็นแบคทีเรียที่สามารถตรึงไนโตรเจนจากอากาศให้อยู่ในรูปของไนโตรเจนที่พืชตะกูลถั่วสามารถนำไปใช้ได้ อย่างไรก็ตามการผลิตหัวเชื้อไรโซเบียมในระดับอุตสาหกรรมขนาดใหญ่มีต้นทุนสูง เพื่อที่จะลดต้นทุนในการผลิตหัวเชื้อแบรดี้ไรโซเบียม สามารถผลิตในระดับอุตสาหกรรมได้โดยใช้ อินฟิวชั่นปั๊มร่วมกับการใช้หัวเชื้อเริ่มต้นในปริมาณน้อย เทคนิคนี้สามารถใช้เป็นนวัตกรรมใหม่ในการผลิตหัวเชื้อแบรดี้ไรโซเบียม งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาประสิทธิภาพการผลิตหัวเชื้อไรโซเบียม และลดต้นทุนในการผลิตหัวเชื้อ โดยการใช้เทคนิคการเจือจางเซลล์แบคทีเรียด้วยเทคโนโลยีอินฟิวชั่นปั๊ม เพื่อใช้ผลิตในการผลิตหัวเชื้อแบบเหลว หัวเชื้อแบบผนมในวัสดุพาหะเช่น พีท (peat) และแบบผสมเม็ดวุ้น (encapsulated bead) ซึ่งงานวิจัยนี้ได้นำเชื้อทางการค้า *Bradyrhizobium diazoefficiens* USDA110 มาใช้เป็นต้นแบบในการทดสอบเพื่อการผลิตหัวเชื้อไรโซเบียมในรูปแบบต่าง ๆ โดยทำการเปรียบเทียบคุณภาพของหัวเชื้อเมื่อทดสอบปลูกถั่วเหลืองในสภาพไร่

จากผลการทดสอบพบว่า เชื้อตั้งต้นที่มีความเข้มข้นต่ำตั้งแต่ 10 ถึง 10^6 เซลล์ต่อหน่วย (CFU/unit) เป็นช่วงที่เหมาะสมสำหรับการเจือจางเชื้อ และจากค่าเจือจางของเชื้อตั้งต้นดังกล่าวสามารถทำให้เชื้อมีการเจริญ และเพิ่มจำนวนเซลล์ได้สูงถึง 10^8 เซลล์ต่อหน่วย ในหัวเชื้อทั้งแบบเหลว หัวเชื้อแบบผสมกับพีท และผสมเม็ดวุ้น นอกจากนี้ พบว่าเทคนิคการใช้ syringe และ peristaltic pumps ทำให้ได้ค่าเจือจางของเชื้อตั้งต้นเท่ากับ 10 เซลล์ต่อหน่วย สามารถนำไปใช้ในการผลิตหัวเชื้อทั้งแบบเหลว หัวเชื้อแบบผสมกับพีท และผสมเม็ดวุ้นได้ แต่ในการผลิตหัวเชื้อในอุตสาหกรรมที่ใหญ่กว่าโรงงานต้นแบบนั้น มีความเหมาะสมสำหรับการผลิตหัวเชื้อแบบเหลว และหัวเชื้อแบบผสมกับพีทเท่านั้น แต่ไม่เหมาะสมสำหรับการผลิตหัวเชื้อแบบผสมเม็ดวุ้น

สำหรับการทดสอบระยะเวลาในการเก็บรักษาหัวเชื้อจากการใช้เทคโนโลยีการผลิต พบว่าสามารถเก็บรักษาหัวเชื้อไว้ได้ระยะเวลานานถึง 6 เดือน ณ อุณหภูมิห้อง และมีจำนวนเซลล์ที่มีชีวิตสูงกว่า 10^8 เซลล์ต่อหน่วย ซึ่งถือเป็นการทดสอบครั้งแรกที่มีการใช้หัวเชื้อความเข้มข้นต่ำเท่ากับ 10 เซลล์ต่อหน่วย แล้วสามารถเพิ่มจำนวนเซลล์ได้ถึง 10^8 เซลล์ต่อหน่วย ในทุกประเภทของหัวเชื้อ ผลการทดสอบคุณภาพของหัวเชื้อในสภาพไร่พบว่า หัวเชื้อทั้ง 3 ประเภท สามารถส่งเสริมการเจริญเติบโตของถั่วเหลือง โดยเชื้อมีการตรึงไนโตรเจน ให้กับพืชได้อย่างมีประสิทธิภาพ และเมื่อ

วัดน้ำหนักสด และแห้งของ ปมถั่ว ราก ต้น และฝัก รวมทั้งปริมาณคลอโรฟิลล์ของต้นถั่ว เปรียบเทียบกับต้นถั่วเหลืองที่ไม่ได้ปลูกเชื้อ พบว่าการปลูกเชื้อทำให้ถั่วเหลืองมีน้ำหนักที่สูงกว่าอย่างมีนัยสำคัญเมื่อเก็บตัวอย่างที่ 30 45 และ 65 วัน หลังปลูก จากผลวิเคราะห์ต้นถั่วที่ 55 วัน พบว่า เปอร์เซ็นต์การสะสมไนโตรเจนในต้นถั่วที่มีการใช้หัวเชื้อ มีค่าสูงขึ้นเท่ากับ 1.98 ถึง 2.05 เท่า และปริมาณไนโตรเจนเท่ากับ 130.07 ถึง 139.1 กิโลกรัมต่อเฮกตาร์ ส่วนค่าการตรึงไนโตรเจนโดยหัวเชื้อ มีค่าเท่ากับ 108.16 ถึง 117.20 กิโลกรัมต่อเฮกตาร์ และพบว่าเมื่อเก็บผลที่ 100 วัน การใช้หัวเชื้อไรโซเบียมมีประสิทธิภาพในการเพิ่มผลผลิตได้ถึง 2.56-3.48 ตันต่อเฮกตาร์ จากผลงานวิจัยในครั้งนี้ ทำให้ทราบว่า การผลิตหัวเชื้อไรโซเบียมโดยการใช้เทคนิคเจือจางเซลล์แบคทีเรียด้วยเทคโนโลยีอินฟิวชั่นปั๊ม สามารถส่งเสริมการเจริญเติบโตของถั่วเหลืองในสภาพไร่ ดังนั้นการพัฒนาเทคนิคเพื่อผลิตหัวเชื้อในงานวิจัยนี้ จึงสามารถนำไปประยุกต์ใช้ในการผลิตหัวเชื้อจำนวนมาก เพื่อให้ได้หัวเชื้อคุณภาพสูง และลดต้นทุนการผลิต โดยเฉพาะในประเทศกำลังพัฒนาได้ในอนาคต

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2563

ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
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BEEDOU APHAISO : THE INNOVATIVE TECHNOLOGY TO PRODUCE LIQUID AND SOLID INOCULANT OF *Bradyrhizobium diazoefficiens* USDA110. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Dr.rer.nat., 103 PP.

SOYBEAN/*Bradyrhizobium diazoefficiens*/INOCULANTS/INFUSION STARTER/ PILOT SCALE

Bradythizobium is bacterium that can fix nitrogen from atmospheric nitrogen to bioavailable as ammonium for leguminous plants. However, bradyrhizobium inoculant has a high manufacturing cost in large scale. Therefore, to reduce the cost of bradyrhizobium inoculant production in large scale, the inoculants could be manufactured using an application of infusion pumps with a small amount of starter culture. This technique could be used as innovative technology to produce bradyrhizobium inoculants. The objective of this research was to develop efficient and low cost *Bradyrhizobium* inoculant production using the incorporation of a dilution technique with infusion pump technology for various inoculants (liquid-, peat-, and encapsulated-inoculant). *Bradyrhizobium diazoefficiens* USDA110 was used as the rhizobial model in this research for soybean inoculant production and field-tested application.

An effective varied and small amount of starter culture from 10 to 10^6 CFU/unit grew and increased the cell population number up to 10^8 CFU/unit in liquid-, peat-, and encapsulation-inoculants. For the pilot scale production, the syringe- and peristaltic pumps were fused to produce small micro-injection starter culture at 10 CFU/unit to produce liquid-, peat-, and encapsulated inoculants. This system can be

used in a large scale liquid-, and peat-inoculant production. Nevertheless, it may not be suitable for a large scale encapsulated inoculant production. The shelf-life of inoculants produced from this developed technology for at least 6 months with a cell population number higher than 10^8 CFU/unit when stored at room temperature. This is the first demonstration of the diluent with a very low concentration of a starter population of 10 CFU/unit that increased the cell number to 10^8 CFU/unit in different types of inoculants. All types of bradyrhizobium inoculants were used to investigate the symbiosis efficiency in the field experiment. Three types of inoculant promoted soybean growth, and N_2 fixation in field conditions. The height, N_2 fixation efficiency, nodule number, nodule dry weight, stem dry mass, root dry mass, pod quantity, pod dry mass, total plant dry mass, and chlorophyll content were significantly higher than that of non-inoculated plants at 30, 45, and 65 days after inoculation (dai). The inoculated plants increased the percentage of nitrogen content in soybean residue (1.98-2.05-folds), total N content 130.07-139.1 Kg/ha, and N-fixed 108.16-117.20 Kg/ha at 55 dai. The bradyrhizobium inoculants effectively increased yield production at 2.56-3.48 ton/ha at 100 dai. These results indicated the inoculant production using the incorporation of the dilution technique with infusion pump technology could promote soybean growth under field conditions. Therefore, this developed technique can be further applied for pilot scale production of high quality bradyrhizobium inoculant in developing countries.

School of Biotechonology

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LIST OF ABBREVIATIONS

˚C	=	degree Celsius
%	=	percentage sign
µg	=	microgram
µl	=	microlitre
µm	=	micrometer
$	=	Dollar
FA	=	Fluorescence Antibody
ANOVA	=	Analysis of variance
BCE	=	Before the Common (or current) Era
CE	=	Common (or current) Era
CFU	=	Colony forming unit
dai	=	days after inoculation
DNA	=	deoxyribonucleic acid
DOA	=	Department of Agriculture
et al.	=	Et alia (and other)
FITC	=	Fluorescein isothiocyanate
h	=	hour
ha	=	hectare
kg	=	kilogram
L	=	litre

LIST OF ABBREVIATIONS (Continued)

lbs	=	pound
M	=	molarity
mg	=	milligram
min	=	minute
ml	=	milliliter
mm	=	millimeter
MPN	=	Most Probable Number
N	=	Nitrogen
OD	=	Optical Density
OROG	=	One Research One Graduate
PBS	=	Phosphate-Buffered Saline
PCR	=	Polymerase Chain Reaction
PVP	=	Polyvinylpyrrolidone
RCBD	=	Randomized Complete Block Design
RFLP	=	Restriction Fragment Length Polymorphism
rpm	=	Rounds Per Minute
SD	=	Standard Deviation
SUT	=	Suranaree University of Technology
USA	=	United States of America
USD	=	United States dollar(s)
w/v	=	weight per volume

LIST OF ABBREVIATIONS (Continued)

YEM	=	Yeast Extract Mannitol
YMB	=	Yeast Extract Mannitol Broth

CHAPTER I

INTRODUCTION

1.1. Significance of study

Bradyrhizobia are involved in the symbiotic with legumes by the colonization and formation of root nodules. The bacteria produced nitrogenase enzyme for fixing nitrogen to ammonium located in 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 is able to fix nitrogen higher than 200 kg/hectare (Denton et al., 2017). In addition, bradyrhizobial inoculant was low cost and can reduce the chemical fertilizer utilization. Furthermore, biofertilizer can help to conserve soil, community of microorganisms, and support sustainable agriculture. To produce rhizobium inoculant for industrial scale, it is necessary to set up many types of equipment in the process and lead to high cost which is difficult for new investors to produce inoculant. The general process to produce inoculant in commercial-scale requires the large capacity for sterilized medium, starter preparation, sampling method, aerating, and cleaning the system (Somasegaran et al., 1985). The main requirement in the process is fermenter to provide an appropriate condition to produce inoculant. In addition, the price of the fermenter is expensive and it is not easy to operate. Besides, a high risk of contamination is normally occurs with a non-experienced producer. Therefore, to start inoculant production, particularly

for the commercial scale is not easy in developing countries. The simple technology and easy production technique are required.

To reduce the cost of large scale rhizobial inoculant production, the dilution technique can be used to reduce the high amount of starter preparation. The dilution method is a basic technique used widely in bio-science. Dilution of liquid rhizobium to peat carriers could increase the production capacity of inoculant legume (Somasegaran et al., 1985). The advantage of the dilution method is to reduce the volume of starter for peat carrier. This process does not require a high volume of medium and a large fermenter. This technique has been used to produce liquid inoculant of rhizobium by starting from a single colony of *Bradyrhizobium diazoefficiens* (USDA110) in yeast mannitol broth medium (YMB medium) 20 ml, and after 7-day the culture was injected into 1-2 liters of glass fermenter (Tittabutr et al, 2001). Once the culture obtains OD_{600} = 1, the culture was transferred into 100 liters of steel fermenter culture to complete the big scale production and final package in bottles (Somasegaran et al., 1985). The price of rhizobium biofertilizer is around 0.5 USD per package in India (Santosh et al, 2012). The price solid inoculant is 0.625 USD (200 g/package) and one bottle of liquid inoculant is 0.312 USD (80 ml/bottle) in Thailand (Department of Agriculture, 2020).

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 (instead bags using a small amount of starter culture to produce inoculant). 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, 1985; Tittabutr et al., 2007). 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. The diluent of starter culture by mixing with sterilized water was 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 is 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.

1.2 Research objective

1.2.1 Main objective

To develop innovative technology to produce high quality of *B. diazoefficiens* USDA110 inoculant

1.2.2 Specific objectives

1. To determine an appropriate infused level for USDA110 starter to produce inoculant

2. To modify the infusion pump for production of liquid-, solid- and polymer entrapped-form of inoculant

3. To investigate the shelf-life of USDA110 during storage

4. To test the efficiency of USDA110 inoculant on promote soybean yield under field experiment

1.3 Hypotheses

1. The very low number of bradyrhizobial cell can be used as starter for inoculant production.

2. Infused technique can be used to produce liquid-, solid- and polymer entrapped-form of bradyrhizobial inoculant.

3. The developed inoculants can prolong shelf-life of USDA110 at least six months at room temperature.

4. The developed inoculants are able to promote soybean growth in the field application.

1.4 Scope of limitations

This study was focused on an innovative methodology that enhances the efficiency of USDA110 inoculants production based on the application of dilution technique together with the infused method using an infusion pump (Figure 1.1). First, the infused of rhizobial starters with high concentration was diluted to 10^6 cells/ml with sterilized distilled water that was used to produce inoculant. Second, the infusion pump was used for injecting starter to liquid-, solid- and cell entrapped-form of inoculants. Third, the shelf-life of USDA110 in three forms of inoculant during storage was investigated. Finally, the USDA110 inoculants were inoculated to promote soybean (*Glycine max*) in the field. The capacity of nodulation from USDA110 could be confirmed by fluorescence antibody (FA) from root nodules.

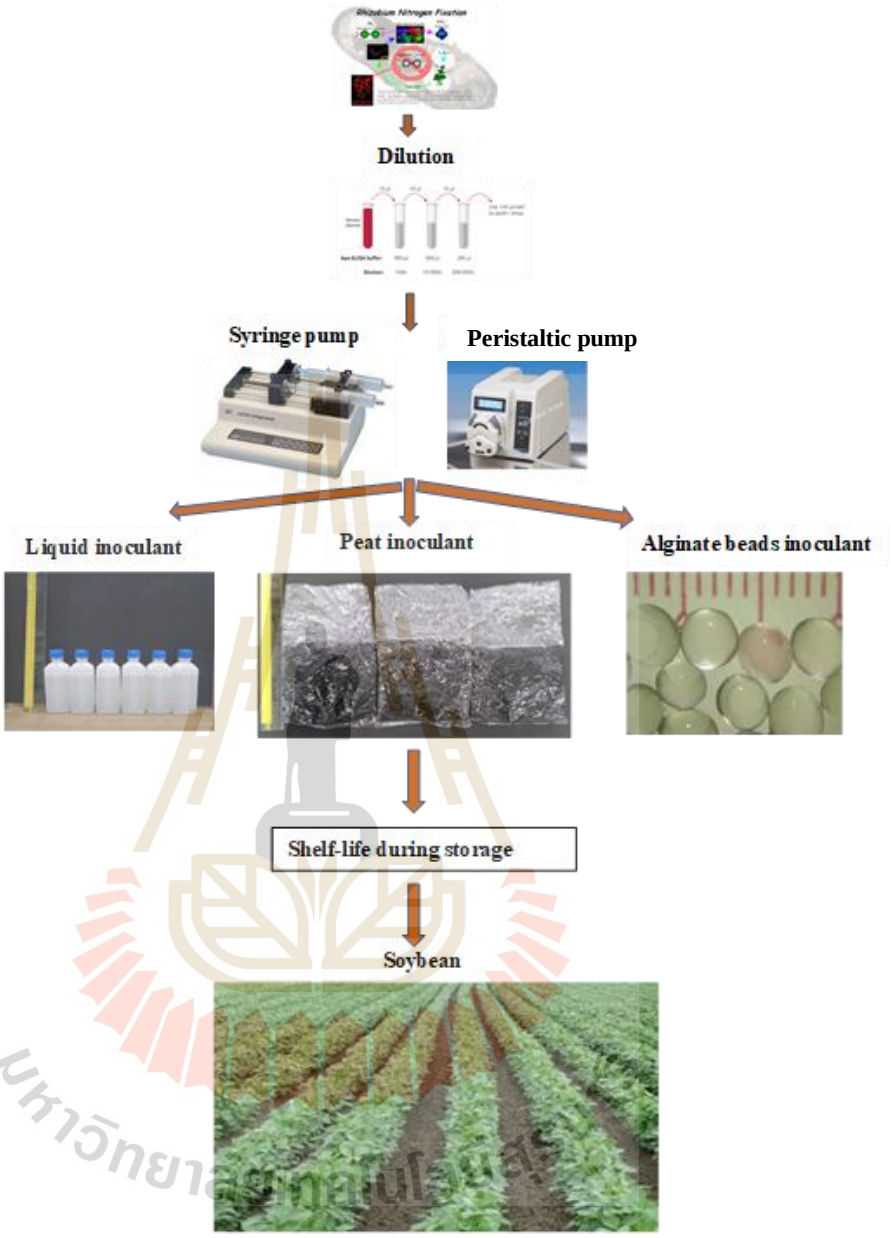

Figure 1.1 The innovative technology inoculant production.

CHAPTER II

LITERATURE REVIEW

2. 1 Interaction rhizobium with legume plant

Rhizobia are bacteria that benefits to legume plant under symbiosis condition. They can convert atmospheric nitrogen to ammonium for the legume and lead to increase high-protein content in legume seeds and forage. This process called biological nitrogen fixation. The biofertilizer is used generally in the world wide to produce food for people and feed for animals. *B. diazoefficiens* (USDA110) was competitively symbiosis with soybean (*Glycine max*) and promoted N-fixation at nodules root. The rhizobium fix nitrogen higher than 200 kg/hectares in soybean (Denton et al., 2017). In addition, rhizobium can highly reduce the chemical fertilizer utilization, which may destroy soil structure in agriculture system. There is low cost when compared with chemical fertilizer. Biofertilizers can help to conserve soil, the community of microorganisms and sustainable agriculture. Especially, rhizobium that is specifically compatible with a particular species of legume can stimulate the formation of effective root nodules and ineffective to increase the yield of legume.

2.2 Biological nitrogen fixation

Biological nitrogen fixation (BNF) occurred by *Rhizobium* sp. that can converted the N_2 from atmospheric to the ammonium by nitrogenase enzyme (Figure 2.1). BNF was firstly reported by the German agronomist Hermann Hellriegel and

Dutch microbiologist Martinus Beijerinck (Paracer and Ahmadjian, 2000). The nitrogenase had two component protein (dinitrogenase and dinitrogenase reductase). The nitrogenases were sensitive with the oxygen (López-Torrejón et al., 2016). The three of dinitrogenase was shared similar structural properties but differ in the heterometal present in the active site of the dinitrogenase unit. The nitrogenase contains iron molybdenum cofactors at the active site and is encoded by the *nif* gene family. Vanadium was cofactor of nitrogenase by iron vanadium cofactor that belong *vnf*-encoded. The alternative cofactor of nitrogenase contains iron only cofactor (FeFe-co) and is *anf*-encoded. The nitrogenase catalyzed to involves the MgATP-dependent reduction of nitrogen gas to two molecules of ammonium (Willing et al., 1989). The equation was shown as follow:

$$N_2 + 8H^+ + 16ATP + 8e^- \Longrightarrow 2NH_3 + H_2 + 16\ ADP + 16\ Pi$$

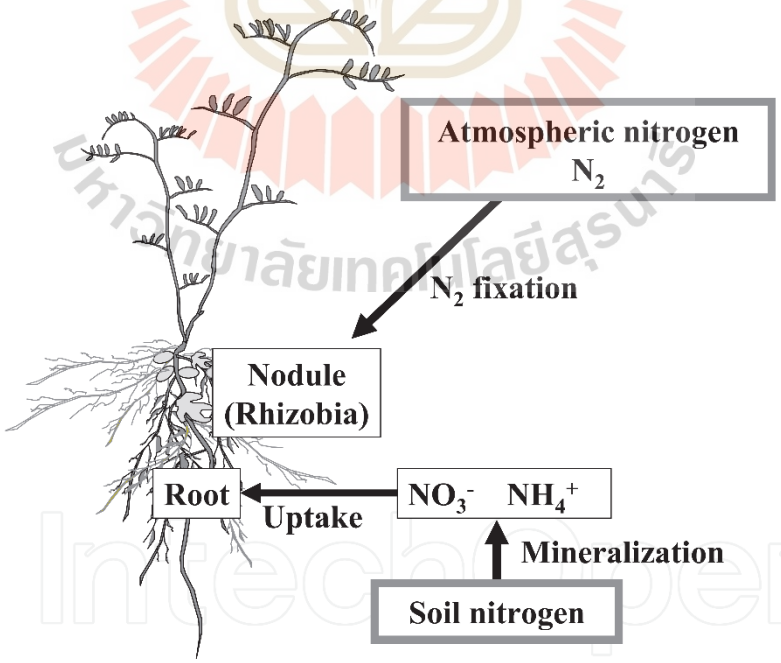

Figure 2.1 Nitrogen flow and utilization of a legume plants (Takashi Sato, 2014).

2.3 The importance of soybean

Soybean (*Glycine max* L. Merr.) is considered to be one of the oldest crops cultivated by humans. It is believed to have originated in China, possibly in the northern and central regions. Some indication indicates that soybean was domesticated as early as 3500 BCE, and was subsequently introduced into Korea around 200 BCE and into Japan and Russia around 300 CE. Yearly world production of soybean is nearly 104.5 billion USD (Yan and Baidoo, 2018). The important countries of the world with the highest rate of soybean production include the USA, Brazil, Argentina, China and India (Masuda and Goldsmith, 2009; Ray et al., 2013). Soybean is a significant source of food, protein, and oil. Recent, more research is critical to increasing its yield under different conditions, including stress. Interest in the impact of agriculture on changing soil species makeup has increased. Due to its key situation as one of the more important crops, more research into management can pay to a better understanding of its production. With respect to the importance of soybean production worldwide, its production must be assessed from different perspectives including its with soil microbes. Many crop species as well as soybean are found related to microbial interactions may have vital functions in soybean production and health. It is similarly important to evaluate the abiotic factors which interact with the growth and yield of this crop (Pagano and Miransari, 2016).

2.4 *Bradyrhizobium diazoefficiens* strain USDA110

Bradyrhizobium greatly plays a special role in the nitrogen cycle of agroecosystems by nodules formation in the roots of soybean (*Glycine max*) and starting dinitrogen-fixing nodules. Thus, significant nitrogen gas is fixed and

transferred to the soybeans to produce the nitrogen fertilizer (Prakamhang, 2013). bradyrhizobium is the predominant genus of rhizobia in the tropics (Delamuta et al., 2012). Tropical regions have diverse environmental gradients that could influence the diversity of organisms. By the way, a high diversity of rhizobia was informed in tropical regions (Delamuta et al., 2012). Amid the interaction between rhizobia and leguminous plants have more detail such as the presentation of nodules or the formation of ineffective ones on soybean roots may be resulting from host micro-symbiont incompatibility. The role of both known and unknown bio-molecule included flavonoids, polysaccharides, and hormones (Daayf et al., 2012). Moreover, the inoculants production and application should be effective with nodules in the soybean under the field condition. Each country may have the standards of law for rhizobial inoculants for controlling the quality of rhizoum inoculant.

There are many diverse species of bradyrhizobia as the micro-symbionts of soybean including *Bradyrhizobium diazoefficiens, B. elkanii, B. yuanmingense, B. liaoningense, B. huanghuaihaiense,* and *B. diazoefficiens* (Delamuta et al., 2013). Therefore, various information showed that soil pH, salinity, climate, nutrients, and cultural management have effectively grown from soybean bradyrhizobia (Saeki et al., 2017). The various diversity of microorganisms in the soil highly supported a significant role in keeping soil health that has increased the yield of soybean.

The *B. diazoefficiens* USDA 110 was isolated by D.F. Weber (Beltsville Agricultural Research Center, USDA-Beltsville Culture Collection. Beltsville, 20705) (Mathis et al., 1986). Under the field of Hawaiian soils, the *B diazoefficiens* strains USDA 110 was more competitive than USDA 123 under the devoid naturalized (Kosslak and Bohlool, 1985). The rhizobium strains CC 709 and USDA 110 were tolerant in

dehydrated soil higher than strains CB 1809 and USDA 123 (Al-Rashidi et al., 1982). *B. diazoefficiens* USDA110 was most commonly used for the inoculation of soybean in many countries in USA, Canada, and South America. The USDA110 was effectively promoted N-fixation in soybean and supported the production of Indole-3-Acetic Acid (IAA), gibberellic acid (GA3) and abscisic acid (ABA) (Boiero et al., 2007). The *B. diazoefficiens* USDA110 was highly nodulated in soybean root with the iron soil (Fuhrmann and Wollum, 1989) and nodulated significant competition and nodule forming (McDermoti and Graham, 1990). The USDA110 was co-inoculated with *Bacillus velezensis* S141 able to promoting nitrogen fixation and soybean growth (Prakamhang, 2013; Sibponkrung et al., 2020). Based on the review, the *B. daizoefficiens* USDA110 was a major strain to the growth productive of soybean in the global.

2.5 Rhizobium inoculant

Rhizobium inoculants are the key to deliver the biological nitrogen fixation, it transports rhizobium to the legume in the field. The legume should be precisely added an appropriate number of rhizobia to ensure the forming of more nodules number and promote legume yield. The decent character of rhizobium inoculant was a comfortable environment for rhizobium. The rhizobium should be tolerant to biotic and abiotic conditions including high temperature, low moisture in the soil, work every type of soil, actively with the pesticide in legume seed, and work well in culturing practices on field. Besides, the inoculant was completed with another microorganism, easily to use, maintain long time survival of Rhizobium inoculated in the soil. When the plant needed inoculant, the shelf-life was protected more than one season and reproducible

results in the ground. Although, the inoculant did not harmfully with human, animal and plant safety by disregarding of hazardous materials (Bashan et al., 2014).

There are three basic purposes for all inoculants including, to (1) encourage the growth of the intended rhizobium, (2) support the number of rhizobia live in good physiological condition for an acceptable period, and (3) transfer enough rhizobium at the time of inoculant to the legume (Stephens and Rask, 2000). The factors that may impact efficiency are provided good survival of the seed. Those include: (1) growth phase at the time rhizobium take a life cell and could work fine in the carriers, (2) the carriers drying and moisture has protected the self-life of rhizobium growth in carriers, (3) optimal of carrier material typical, and inoculant technology (Date, 2001).

2.6 Types of rhizobium inoculant

There are five types of Rhizobium inoculant as follow: (1) Soil-based inoculant: (peat, coal, clays, and inorganic soil), (2) Agar inoculant, (3) Polymers inoculant, (4) Lyophilized inoculant and (5) Liquid inoculant (Figure 2.2).

2.6.1 Agar inoculant

This agar inoculant is the first presence formulation with the commercial inoculants in the USA (Nobbe and Hiltner, 1896). It was formed on gelatin, and later on agar. The agar inoculant was used directly to the seed. This method was easily used and effected completely legume plant inoculation. The major disadvantage of agar inoculant is the high mortality rate during the drying phase immediately following the application to the seed. This mortality could decrease significantly by the addition of 9% (w/v) maltose to the suspending liquid (Brockwell, 1982). However, agar using with alginate and perlite that can be used for cell

immobilization materials to prolong the survival of the inoculant strains. The *Ensifer* spp. strains LP2/20, NK2/9, and *Bacillus* sp. strain NK2/17 were selected, immobilized, and stored at 30°C for 15 days. After 15 days of incubating, *Ensifer* sp. strain LP2/20 immobilized in agar highly increased the number of bacterial survivors which was significantly different from other resources (Nimnoi et al., 2017).

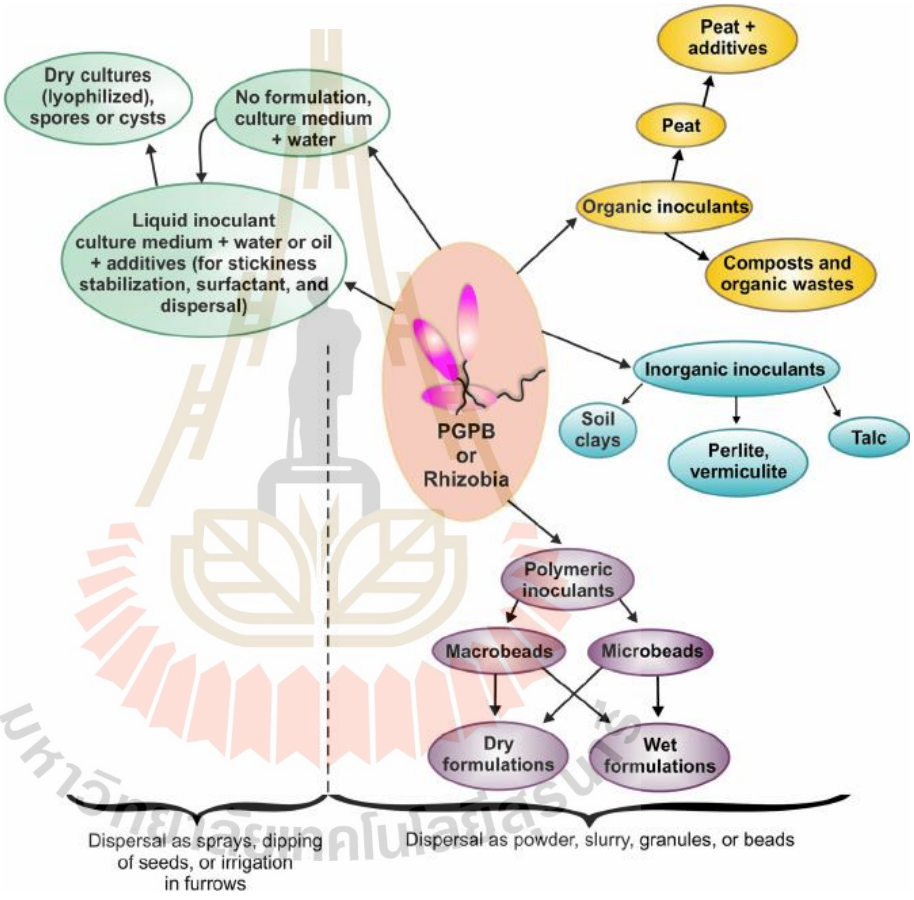

Figure 2.2 Types of inoculant (Bashan et al., 2014).

2.6.2 Soil-based inoculant

Soil-based inoculant was highly used around the world market and the main basic encouragement inoculant was peat. Another soil-based inoculant source was used other such bagasse, cork compost, attapulgite, sepiolite, perlite and amorphous silica (Figure 2.3) (Albareda et al., 2008). However, some carriers were not appropriated suitable for rhizobium growth and keep shelf-life by great type of physical and chemical. The low quality of carriers were transferred the low number of rhizobium to plant and low infected the nodulations in legume root (Somasegaran and Hoben, 2012). The appropriate physical and chemical properties of peat to make inoculant are high water holding capacity, non-toxic to rhizobium, easy to sterilize by autoclaving or gamma irradiation, willingly and cheaply available, deliver good adhesion to seed, have pH buffering capacity and have cations and or anions exchange capacity. For profitable purposes, a safe storage of 6 months can be protected from peat-based inoculant (Albareda et al., 2008).

The procedure to formulate inoculants, initiated by collection and screening to take away debris, such as stones, roots and formerly drying by air the temperature should not more than 100°C. The higher temperatures can degrade peat and release toxic substances. Peat with a particle size of 10-40 μm is composed of seed coating, and peat with the particle size of 500-1500 μm is used for the production of soil implant (granular form) inoculant. Most peats are too acidic. Therefore, peat was adjusted by $CaCO_3$ (pH 6.5-7.0). The sterilized and non-sterilized forms are used in commercial production systems. Peat could be sterilized by autoclaving at 15 lb/in pressure, 121°C for at least 60 minutes. Therefore, Gamma-irradiation sterilization was chosen. The sterilization of peat supported high number of rhizobium, but more

costly than non-sterile peat. Therefore, the non-sterilized peat is offered in commercial legume inoculant as well (Figure 2.3) (Tittabutr et al., 2001).

Figure 2.3 Solid inoculant producted by compost (https://www.limogesseedfarms .com/who-we-are-1-2-2-1-2).

2.6.3 Liquid Inoculants

Liquid inoculants are "no-formulation". Fundamentally, they are microbial cultures or suspensions amended with substances that may advance stickiness, maintenance and surfactant and dispersion capabilities (Singleton et al., 2002). Liquid preparations allow the producer to include enough amounts of nutrients, cell protectants and inducers responsible for cell/spore/cyst formation to progress performance. Liquid inoculants contain a concentration of 2×10^9 cells/ml, allowing for lower application rates and increased efficiency in using inoculants (Schulz and Thelen, 2008) (Figure 2.4). Additionally, it is no contamination and has a long-time shelf-life for some formulations, better safety against ecological stresses, and improved field efficacy, associated with peat-based inoculants (Singleton et al., 2002). They are well-matched with equipment on large farms, such as air seeders and seed

augers. Several compounds can be possibly used as additives to generate liquid inoculants, such as glucose (Tittabutr et al., 2007), carboxymethyl cellulose, glycerol, polyvinylpyrrolidone, trehalose, FeEDTA, and gum arabic (Wani et al., 2007).

Liquid inoculants can be added more polymers to the broth culture. These polymers are used to keep bacterial cells from stresses and help their distribution to the host (Albareda et al., 2008). Their problem is not stored at room temperature for a long time that is lost the viability of the bacteria. Now, the physical and chemical properties of polymers protect cells in contradiction of desiccation and sedimentation, which is related to cell death (Rouissi et al., 2011; Sivasakthivelan and Saranraj, 2013) (Figure 2.4). The life of the *B. elkanii* strains in polymer inoculant was used in the mix biopolymer carrier xanthan, polyvinylpyrrolidone (PVP) and Jatai gum, the amount of rhizobium could be maintained more than peat throughout 8 months

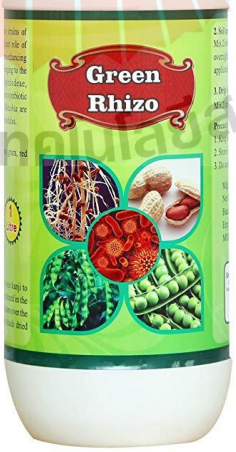

Figure 2.4 Liquid inoculants (https://www.amazon.in/Green-Rhizo-Fertilizer-Rhizobium-japonicum/dp/B01H34MWOC).

of storing (Denardin and Freire, 2000). Liquid inoculants formulated with Gum Arabic at 0.1%, glycerol 0.5%, polyvinyl pyrrolidone 0.5%, polyethylene glycol 0.5%, alginate 0.1% gum Arabic 0.5% to encourage long term storing of rhizobium and bradyrhizobium for 6 months (Sethi and Adhikary, 2012).

2.6.4 Polymeric encapsulated inoculant

Synthetic formulations of polymers have been continuously assessed for decades; they offer more advantages over peat (Figure 2.5). These include longer shelf life, good persistence at the destination field, necessary cell density, ease of work and improved suitable in general (John et al., 2011). For farming and environmental uses, these polymers include, so far, alginate, agar, pectin, chitosan, bean gum and proprietary polymers. The bacteria were mixed with a gelling compound (adjuvant) in a polymer solution. The polymer solution was additives such as bran, peat, charcoal, clay, and others that drop-wise into a calcium salt solution to solidify and form the gel pellets. The size and shape can be precised by variable the drop-forming structure. The particles are then dehydrated for ease of packing and management. From commercial and agricultural positions, longer survival of bacteria in these polymeric production makes dry formulations various attractive. Many researchers conclude the dried polymer have been the good outcome with bacteria and plant the seed, the dry seeds of wheat, basil, cabbage and radish coat alginate or bran and chitin additive without PGPR did not affect with seeds germinate (Sarrocco et al., 2004).

Microbeads alginate beads were satisfactory more than 10^{11} (CFU) g^{-1} inoculant for seed inoculation (Bashan, Hernandez, Leyva, & Bacilio, 2002), The alginate mixed with clay was enlarged the number of cell unit establishing 1.1 to 1.3 log g^{-1}. However, the major disadvantage of polymeric inoculants is that the raw

material for all polymers are expensive when compared to peat, soil and organic waste inoculants.

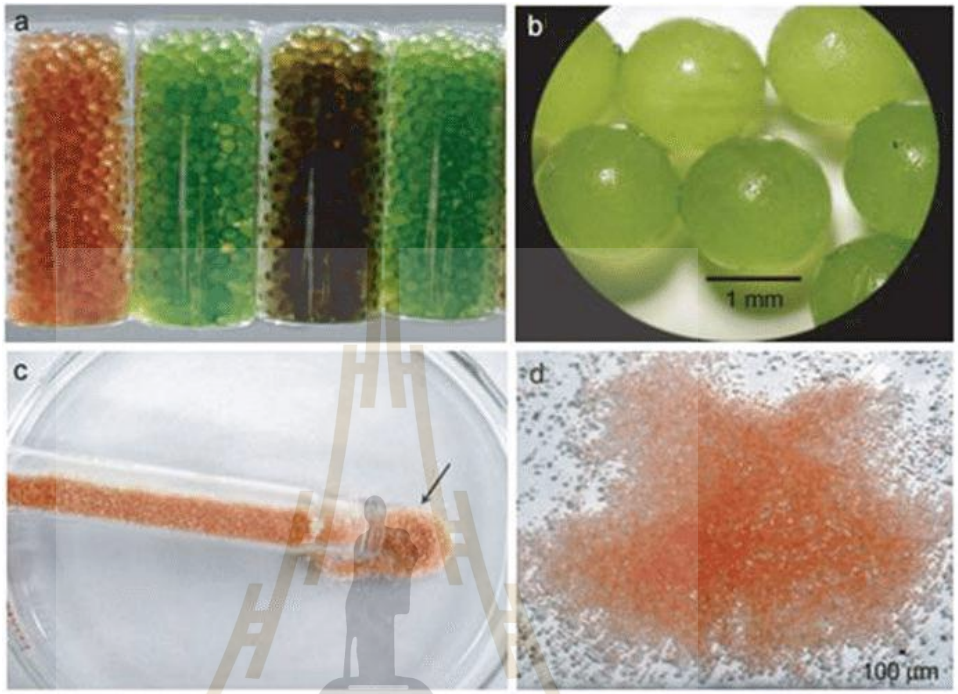

Figure 2.5 Polymeric inoculant, (a, b) wet macrobead inoculant and (c, d) dry microbead inoculant (Bashan et al., 2016).

2.6.5 Lyophilized inoculant

Lyophilization was used capturing bacteria for agricultural and environmental usage. The encapsulation was protected high cell bacterial under environment, but the number of bacteria was regularly decreasing during storage. The lyophilized cultures could deliver good nodulation in the field. Therefore, the lyophilized rhizobia on the seed is poor (Vincent, 1965). A microbead preparation containing *A. brasilense* was air dehydrated at 38°C, suitable a powdered substance. Each bead contained more than10^{9}CFU g^{-1} bacterium. Otherwise, dry microbeads

were formed using a standard freeze-drying process. The effectiveness of freeze-dried alginate beads was confirmed with an agricultural strain of *Pantoae agglomerans*. The dry beads were mixed with bacteria, glycerol and chitin. Glycerol increases hole size within the beads, which affects the slow-release properties, where the addition of glycerol and chitin improved survival throughout the freeze-drying process. These beads were able to keep the applied *P. agglomerans* strain IC1270 (PGPB) to the soil compared to bacterial suspension (Zohar-Perez et al., 2002).

2.7 Advantage and disadvantage of each inoculant type

Table 1.1 The advantage and disadvantage of each inoculant type.

Formulations	Advantage	Disadvantage	References
Agar inoculant	- This method proved easy and successful that, with modifications, it was used for all legume plant inoculation.	- The major problem of agar inoculant is the high-volume rate during the drying phase immediately following application to the seed	(Nobbe and Hiltner, 1896)
Soil-based inoculant	- Peat is optimal carriers of rhizobium, to defend self-life by good distinguishing of physical and chemical properties	- The production of peat inoculants is complex and need many steps	(Brockwell, 1982)

Table 1.1 The advantage and disadvantage of each inoculants type (Cont.).

Formulations	Advantage	Disadvantage	References
	- There is delivery highly amount of rhizobium to legume, and increasing number of nodulations in legume root - The appropriate physical and chemical properties of peat to make inoculant should be high water holding capacity - Non-toxic to rhizobium, easy to sterilize by autoclaving or gamma irradiation - Be readily and inexpensively available, provide good hold to seed, have pH buffering capacity, and have cations and or anions exchange capacity - For trade purposes, a safe storing period of 6 months	- Heat sterilization of some peat has been found to produce undesirable changes and to release toxins - Non-sterile peat occurs some contaminant by other microorganism	
Liquid inoculant	- They are easy to handle and can be used as a seed inoculant or for distribution directly into seed bed	- Essentially because of the problems which rise in preserving biological control after the cultures leave the manufacturer	(P Somasegaran and Hoben, 2012).

Table 1.1 The advantage and disadvantage of each inoculant type (Cont.).

Formulations	Advantage	Disadvantage	References
	- Enough amounts of nutrients, cell protectants - Inducers responsible for cell/spore/cyst formation to improve performance. - This form of liquid inoculant has good storing physiognomies in the bottle or on the seed - Adheres stubbornly to the seed coat without the need for adhesive - And gives rise to nodulation and N_2 fixation as good as can be gotten with peat inoculant.	- Manufacturers have overcome the problematic of deterioration by concentrating the broth inoculant with centrifugation, placing it in plastic containers, freezing it, and transporting it to the user in a frozen state packed in dry ice. - Limited shelf life in some case - Cold conditions are required for long-term storing - Increased costs and limits for developed countries	
Polymeric inoculant	- Non-toxic and free of harmful preservatives that affect bacteria within the inoculant and inoculated plants	- They have the disadvantage that the best results are obtained when they are preserved in their 'wet' condition	(Bashan et al., 2002)

Table 1.1 The advantage and disadvantage of each inoculant type (Cont.).

Formulations	Advantage	Disadvantage	References
	- Slowly degradable in the soil by soil micro-organisms, thus slowly releasing the bacteria in the needed quantities, usually at the time of seed germination and seedling emergence - Provide physical protection for the inoculated bacteria from soil contestants and numerous environmental stresses - Contain appropriate water for survival of the bacteria - Dispersible in water to allow movement of the bacteria from the polymer to the plants - Able to store dried at room temperatures for long time - Offer a consistent batch quality and a better condition for the bacteria - Able to operat easily giving to the wants of specific bacteria	- Drying caused significant loss of viability of the same order as in dried peat - Drying polyacrylamide-entrapped *Rhizobium* looked to be harmful since nodulation and total N of aerial parts were markedly decreased when dried - Dried PER, alginate-entrapped *Rhizobium* (AER) and xantan-entrapped *Rhizobium* (XER) formulations were of poorer quality	

Table 1.1 The advantage and disadvantage of each inoculants type (Cont.).

Formulations	Advantage	Disadvantage	References
	- Able to add amended with nutrients to advance short-term survival of the bacteria upon inoculation, which is essential to the success of the inoculation process - The units are then dried for ease of wrapping and conduct		
Lyophilized inoculant	- Lyophilized cultures can be provided high nodulation in the field	- This is probable to be restricted to favorable conditions, as survival of lyophilized rhizobia on seed is poor	(Vincent, 1965)

2.8 Inoculation techniques

Rhizobia directly inoculated on to the seed surface or into the soil. Seed applications have an optimum number rhizobium to soil to comparison with soil inoculant. Each technique has good and bad, depending on the necessities for specific inoculation, the type of seeds, the pesticide in the seed, and the amount of inoculant. For example, inoculation of large seeds in a large-scale process involving many metric tons of seeds and needs a dissimilar method to that required for sowing pasture seeds

where only a few kilograms of seeds are obligatory. The methods need to address inoculation under adversative situations, such as in high temperature contact of an air seeder, quick-drying when the inoculant is sprayed into sowing machinery, when inoculated seeds are sown under hot, dry environments, or when seeds are coated with fungicides and herbicides (Date, 2001).

2.8.1 Seed inoculation

Seed inoculation is the most universal and applied inoculation technique. It is easy to use in the field and requires a relatively small amount of inoculant. There are many small differences of the basic technique. Using machine coating was showed variation in the basic technology of seed-coating that has not transformed for decades (Deaker et al., 2004). For a short time, prior to sowing, seeds are powder with peat inoculant, with or without water or adhesive. For small seeds, this is followed by superfine, ground limestone, with or without adhesive, and allowed to dry. Drying can be done in a situation or when the coating is applied before sowing. The seeds, held in shallow trays, are airdried or dried by forced air. Coating and drying using fluidized beds where the seeds are floated on a cushion of pressurized air and then sprayed with inoculant and later coated with ground limestone have proved, at least for rhizobia, less successful. Good pellets are evenly coated with limestone, are dry and without loose limestone on the surface, have good physical fullness, and are fixed enough to resist soft influence when dispensed in seeding machinery (Lyons et al., 2001).

The inoculant is coated with seeds using hand, that system is cheap to operate, but the large scale should have machine added, or mechanical tumbling machines (Schulz and Thelen, 2008). Big farm much working use automated seeders

fitted with an inoculant tank, pump and a mixing chamber commonly used for applying chemical coatings. As these are not compatible equipment for microbial inoculation, the inoculant may separate from the seeds. Because, every seed needs to be coated with many numbers of bacteria, adhesives are used. Adhesives include gum arabic (Wani et al., 2007), carboxymethyl cellulose (Viji et al., 2003), sucrose solutions (Cong et al., 2009), vegetable oils (Bashan et al., 2002), as well as any non-toxic commercial adhesive to the bacteria and seeds. The second function of an adhesive is to prevent the inoculant (either dry inoculant as a powder or wet inoculant once the moisture evaporates) from removing the previous sowing with the seeding equipment, especially the powdered type when applied with air-seeders. Sometimes, the beading of seeds with superfine limestone ($CaCO_3$) is added to balance the acidic nature of the soil. This can be completed with an additional adhesive layer under the lime coating (Deaker et al., 2004). The seeds are then spread with common seeding equipment. It is commonly decided that one essential condition to seed coating is adding an adhesive coating. Thus far, there is no promise on the greatest adhesives. Individually manufacturer empirically estimates which adhesive top fits seeds and inoculants (Cong et al., 2009). As soon as seeds are coated with liquid inoculant that adds or not add with adhesive, the inoculant is directly sprayed into the seeds. Subsequently drying, the seeds are sown.

2.8.2 Soil inoculation

This technique is largely used to apply a bacterial to the field soil. Even though the standards for rhizobia are usually used the similar $CFU \cdot ha^{-1}$ as they are for inoculants inoculation to seeds. The application of high quantities by soil inoculation takes away several constraints. Normally, granular inoculants have size 0.5-1.5 mm

that bead is placed in the seedbed under, above, or together with the seed at sowing time. Granular inoculants are free-flowing when applied by seeding machinery. Granules are easily applied more than powder inoculants, which granular is advantage inoculation to optimal of microorganisms in the field (Xavier et al., 2004). There are several key advantages to granular inoculants. This technique can be avoiding damage to seed coating and prevent the inoculant from adverse effects of pesticides and fungicides coating on seeds. The physical separation between the inoculant and the seeds is overcome with the emergence of the seedling roots growing into the adjacent inoculant. Inoculation of the soil reduces the risk of losing part of the inoculant when the seeds pass through the seeding machinery. Small seeds benefit from soil inoculation because they can be exposed to higher concentrations of inoculant than with seed-coat inoculation. The granular inoculant is optimal with developing countries because when using machinery to separate granular inoculant to the field. The machinery can apply to other application fertilizer, pesticide, inoculation. Nonetheless, this method is not appropriate for the third developing countries, because it uses high price to buy machinery for the small farm.

The granular formulation of peat, marble, biochar, perlite, charcoal, soil mix is appropriate for soil inoculation. This technique can assist the inoculant closely with the plant root and easy to infection with the roots. Slurries of peat inoculants and liquid inoculants were effectively applied to soil or hydroponic systems. There are also drawbacks when inoculating soils, mostly methodological. The large inoculation of soil needs special equipment for increasing the costs. Larger quantities need more storage area and transport, which also increases costs. The technique of choice depends on the availability of equipment, seed size, the fragility of the seed coat and

cotyledon, presence of fungicides applied with the seeds, convenience for the grower, and the cost that the grower is paying for the inoculant (Deaker et al., 2004).

2.9 Production of legume inoculant

High quality of inoculant production included three main factors to release in a good product. There are (1) the quality and processing of the carrier, (2) the purity and efficiency in nodulation and N_2 fixation of the culture, and (3) achieving adequate cell numbers in broth culture and finished product. However, to achieve a good quality of inoculant, four important steps of inoculant production must be considered.

2.9.1 Strain selection

The good rhizobial strains are very important for product quality of inoculant. Rhizobium must have competitive with the type of legume for nodulation, and should efficient N_2 fixation when nodule formation. There is a necessity to choose strains for precise geographic, soil and environmental regions. The appropriate strain(s) for a given market location must be compatible with the commonly grown crop cultivars in that area and display the flexibility to be operative and efficient over a range of geographic and environmental conditions. This adaptability is an understandable requirement since soil types, moisture shapes and cultivar preferences can alter over small geographic areas (Lynch, 1983). Each rhizobium specie has a best for each physical or chemical factor and its growth, or growing the number of populations in the plant host and promote high yield of the legume. Additionally it has been competing with others microbial in the soil, and resistant by soil texture, structure, aeration, pH, temperature, and obtainable moisture, nutrient status, organic

matter content, and weather. Rhizobium can vary greatly adapt to environmental stress (Boonkerd and Weaver, 1982).

2.9.2 Culture production

Medium is the main factor for cultural rhizobia. The composition includes carbon source, nitrogen source, K_2HPO_4, $MgSO_4.7H_2O$, $CaCO_3$, and other chemicals. The medium of the inoculant needs to enhance the number of rhizobium to 10^9 CFU/ml for standard to sale in the market. Starter or liquid inoculant can coat directly on legume seed previously growing seed in the field. Nonetheless for another culture rhizobium, it uses for the starter of seed dry, solid-based inoculant, agar inoculant, polymer inoculant, and organics inoculant. Peat based inoculant is used in large volume for manufacturing, first sterilized peat-based products. The mother culture is diluted with sterilized water to make the number of mothers culture 10^6-10^8 CFU/ml and injection into the sterilized peat (Somasegaran and Halliday, 1982). Besides, culture medium was used the commerce waste as molasses, wastewater form fish oil, wash water milk factory, spend water (Rebah et al., 2002), but it is difficult to control the quality of the substrate for making the medium.

2.9.3 Carriers

There are various carriers such as peat, coal, charcoal, soil clay and plant material. The main of quality to produce inoculant is peat, it is highly suitable for the growth of rhizobium (Albareda et al., 2008). The inoculant was used easy for inoculant and cheeped cost for material (Smith, 1992). The carrier should have two essential properties; it must promote the growth of the rhizobium populations of inoculant strains and prolong shelf-life in the carriers. The carrier should have high water holding capacity and retention characteristics, display chemical and physical uniformity, and be non-toxic to inoculant strains and environmentally friendly. The

carrier should easy to be sterilized by autoclaving or gamma irradiating, similarly it should readily available and inexpensive. The carriers have pH buffering capacity, sufficiently adhesive for effective application to seed, and has the property of cation-anion exchange capacities. It requests also to permit growth after the introduction of the rhizobia, has an acceptable pH, rapidly releases organisms upon use and be in abundant supply (Keyser et al., 1993). Peats achieve the bulk of these requirements; however, the supply of this peat is becoming an important problem in many countries. Even many materials have been used as the carrier, often the ability of an alternative carrier, or entrapment methodology. To sustain organism growth is marginal and also cost consistency of supply, and quality against the adoption of these alternative materials (Rao, 1983; Thao et al., 2002).

2.9.4 Finished product and inoculant shelf life

The legume crop is regularly using inoculants form peat or other materials. The inoculant can promote nodulation during storage and manufacturing, it is usually required. The protection of inoculant shelf-life is very important for inoculant quality, uncertainty previous storage some inoculant can lose the ability. Therefore, the most general solutions to this fundamental problem of extending survival time are reducing moisture in the formulation, dry formulation of inoculant is extremely drying in any a fluidized bed, air-dried, or lyophilized, or store at cooler temperatures. In fully dry formulations, bacteria continue in a sleeping form, its metabolism is actually slow or even stopped, and are resistant to environmental stresses, insensitive to infection, and are more well-matched with fertilizer application. The main trouble with most methods is the persistence of the microorganisms throughout the drying process and storage, increasing death >90% of

the initial incorporated population. The desiccation phase is maybe the most serious and the most stressful for microbes through the preparation process. Further stress condition is throughout the bacteria at the time of inoculation, which products is hydration pressure on the cells. Persistence is highly affected by numerous variables. The bacteria need increasing culture medium for used, the time for collection the bacteria should be actively bacteria cell when harvested from the medium, the procedure of cell immobilization, the use of defensive materials, the kind of drying method, and the degree of dehydration. If correctly dry, the shelf-life of the dried formulation is abundant longer than any moist product. Drying during production of inoculant is a crucial step. The highest passing rate occurs also soon after manufacturing, while in storing, or directly later application to the seeds or soil (Date, 2001).

The moisture content peat is between 40-50% that optimal for growth and survival of a range of rhizobia (Deaker et al., 2004). Accordingly, a practical solution, for example, to recover survival of rhizobia on seeds is a short curative period of 15 d at 25°C or an even extended curative time of up to 120 days, which is improved. This curative favor adaptation of rhizobia to the carrier and risk tolerance to drying (Albareda et al., 2008). Completely this occurs because rhizobia are metabolically lively and as lengthy as nutrients and favorable environmental conditions succeed, they continue increasing inside the inoculant. As soon as the bacterial number of failures from dehydration when the carriers were low moisture content. Temporary storage is highly affected by hot temperatures. Using many types of organic, inorganic, and polymeric formulations, rhizobia in these inoculants continued unaffected for 90-120 days of incubation. Inoculants included of two clays

preserved a high bacterial population for further than 5 months (Albareda et al., 2008). Peat inoculant store good condition that can protect shelf-life more polymerized and synthetic inoculants. Longer-dated of storing was confirmed on rhizobia and PGPB. Dry alginate beads kept for 1 year at room temperature reserved significant growth raise effects on sorghum plants although the populations of *A. brasilense* inside the dry beads failed with time (Trejo et al., 2012).

In conclusion, a real formulation must preserve, over satisfactory phases of time, sufficient feasible bacteria to ensure effective seed inoculation. Elongated shelf-life can be obtained by also growing the amount of rhizobium in the inoculant, so even within weakening in inhabitants over time, satisfactory cells remain active at seeding time. Otherwise, use a preservative in the formulation to rise growth during storage or preserve cold storing that decreases the rate of failure in bacteria. In this situation, level formulations with minor initial inhabitants can be acceptable (Xavier et al., 2004).

2.10 Detection of rhizobium

One of the processes that important for inoculant production is the quality control of rhizobium. The strain of rhizobium using as inoculant must be verified during inoculant production and monitored in the product or after application in the field. Therefore, the bacterial identification technique should be considered. There are several techniques can be used for bacterium identification.

2.10.1 Antigen-Antibody based technique

The antigen-antibody compound was spotted under the microscope by the fluorescent antibody (FA) performance. This technique was to sign and classify

microorganisms concurrently. FAs were beneficial aimed at rhizobial straining documentation in the biological examination. The stage products FAs, antisera were disinfected through ammonium sulfate precipitations and dialysis. The contentment satisfied of the dialysate was determined. The immunoglobulin segment was conjugated with fluorescein isothiocyanate (FITC). The FITC-antibody conjugated was detached after the unreacted FITC by column chromatography (gel filtration). It was tested to classify rhizobia in nodules by the through FA procedure. A fluorescent antibody technique, the rhizobium sample was a thin smear and heat fix. Then smear was covered with 1:10 diluted rabbit antiserum, and incubate for 20 min. Further, the excess antiserum temporarily rinsed with phosphate-buffered saline (PBS). The smear covered with FITC conjugate of goat anti-rabbit globulin and incubates 20 min. After that excess FITC conjugate rinsed with PBS for 20 min. FITC washed with distilled water in 10 min. Finally, the sample dried by air and detected under a UV microscopy. There was numerous techniques identity of rhizobia such as somatic agglutination reaction with a pure culture of rhizobium, agglutinating antigens from root nodules, and performing rhizobial antigen-antibody reactions through gel immunodiffusion. However, there are including the determining strain occupancy of soybean nodules in gel immunodiffusion, producing and applying fluorescent antibodies, identifying rhizobia through the indirect enzyme-linked immunosorbent assay, and identifying rhizobia by immunoblot. The advantage of detection are isolating spontaneous antibiotic-resistant mutants of rhizobia, analyzing nodule occupancy using an antibiotic-resistant marker and distinguishing between strains of rhizobia by Rhizobiophage susceptibility (Somasegaran, 1985).

2.10.2 DNA based technique

The advances in microbiology lengthways with the wealth of refinements in contemporary molecular biology techniques in the previous several years have significantly influenced the investigation of the genetics of rhizobia. The importance and application of a range of molecular genetic techniques and nucleic acid hybridization-based assays have been contributory in the physical location, cloning, and analysis of genes involved in the symbiotic collaboration between rhizobia and legumes. Also, with the application of molecular biology, an improved and stronger picture is now beginning to emerge on the taxonomy and classification of the rhizobia. Restriction enzyme digests of rhizobial genomic DNA and restriction fragment length polymorphism (RFLP) analysis applying precise gene probes have become useful in learning genetic variety and in strain identification for biological studies. There are many techniques of genetic technique for rhizobium such as examining plasmid profiles of *Rhizobium* spp. By an adapted Eckhardt vertical gel electrophoresis technique. Isolating and purifying genomic DNA of rhizobia used a significant method. Isolating and purifying genomic DNA of rhizobia used a quick small-scale method. Digesting a purifying genomic DNA of rhizobia was used restriction endonucleases. Restriction fragments of genomic DNA was separated by horizontal agarose gel electrophoresis. Transporting electrophoretically separated DNA from agarose gels to a membrane by southern blotting. Preparing a DNA probe was noted the *nif* genes on symbiotic plasmids of *Rhizobium* spp. Including a nonradioactive label into a DNA probe by nick translation and using a non-radioactively labeled *nif*KDH gene probe to locate complementary sequences of

rhizobial DNA immobilized on membranes (Flores et al., 1987; Somasegaran, 1985; Somasegaran and Hoben, 1994).

The specific genomic fingerprints were proposed as diagnostic tools by means of amplification of interspersed repetitive DNA sequences present in bacterial genomes and used as rep-PCR (Rademaker, 1997; Versalovic et al., 1994). BOX-PCR was analyzed patterns of DNA by agarose gel electrophoresis, box-PCR fingerprinting, and taxonomy based on the sequencing of the 16S rRNA gene (Binde et al., 2009; Selenska-Pobell et al., 1996). DNA fragments from PCR amplification of random segments of genomic DNA with a single primer of arbitrary nucleotide sequence RAPD analysis (Elboutahiri et al., 2009).

2.11 Production cost for rhizobium inoculant

Pilot-scale production of Rhizobium biofertilizer technology, the cost to produce biofertilizer production through industry is also presented. Investment of 5,000 USD (Table 1.2). A fixed cost, recurring expenses approximately 460 USD per year (Table 1.3, 1.4). The indirect cost towards salary and wages (Table 1.5) packets of biofertilizer can be produced. By selling the biofertilizer 0.5 USD per pack (from India), the net benefit is 6000 USD per year, (Sethi and Adhikary, 2012). The price of rhizobium inoculant in Thailand was low, solid inoculant one package is 0.625 USD (200 g/package), and liquid inoculant for one bottle is 0.312 USD (80 ml/bottle), Department of Agriculture, Thailand. Therefore, we need the new technology to reduce the cost of production.

Table 1.2 Fixed cost for produce biofertilizer (Sethi and Adhikary, 2012).

Equipment Cost	**Cost (USD)**
Glass fermenter (fabricated with aerator), 2 nos.	3,000
Autoclave (one electrically operated), 1 no. 20 L capacity	600
Glass double distillation set 5 L/hr capacity, 1 no.	200
Incubator, 1 no.	400
Compound microscope (binocular), 1 no.	300
Refrigerator, 1 no.	200
Inoculation chamber with UV. 1 no. (fabricated)	100
Polythene sealer, 1 no.	100
Chemical balance, 1 no.	100
Total	5,000

Table 1.3 Recurring expenses per year to produce biofertilizer (Sethi and Adhikary, 2012).

Material	**Quantity**	**Cost of the material (USD)**
Carrier material	10 quintals	80
Broth	1000 L, $0.25 per liter	250
Polythene bag	30000, $1 per 1000 bags	30
Recurring expenses per year	(consumables) plastic/polypropylene bottle + plastic wares + glass wares (conical flask, pippetes, test tube, measuring cylinder, beaker etc.)	100
Total cost		460

Table 1.4 Chemical requirement to produce Rhizobium inoculant (Sethi and Adhikary, 2012).

Chemical required	**For 40 L (g)**	**Total cost for 40 L (USD)**
Mannitol	400	7.5
Yeast extract	16	1.0
NaCl	4	0.5
$MgSO_4$. $7H_2O$	8	1.0
K_2HPO_4	20	1.0
Total cost		11.0

Table 1.5 Salary for labors to produce Rhizobium inoculant (Sethi & Adhikary, 2012).

Salary component	**Value (USD)**
One Microbiologist (skilled) per month	200
One production assistant (unskilled) per month	100
Miscellaneous expenses per year (Maintenance of equipment, fuel charge, office expenses etc.)	100
Total cost	500

Many researchers want to modify the low cost of medium for culture rhizobium such as cassava starch (Tittabutr et al., 2001), leaching liquor of corn stalk (Zhang et al., 2015) and diary sludge 60% can growth all stain of rhizobium (Singh et al., 2013).

2.12 Inoculant Technology

2.12.1 Production of rhizobium culture in fermenters

Glass fermenters are volume 4 liter, its small scale to produce inoculant to use in the laboratory, but it not compatible with commercial to have a big scale. Commonly, glass fermenters can provide suitable conditions to culture rhizobium such as easy to sterile medium in an autoclave, air supply, check contaminant, perform the number of rhizobium to needed (Somasegaran, 1985) (Figure 2.6). The first step of glass fermenter in produce inoculant. The starter was cultured on yeast mannitol both (YEB) and shaked 180 rpm at the room temperature 25-30°C after growth 7 days. Further, the starter culture was inoculated to 2.5-liter YEB of Erlenmeyer flask. The cotton-

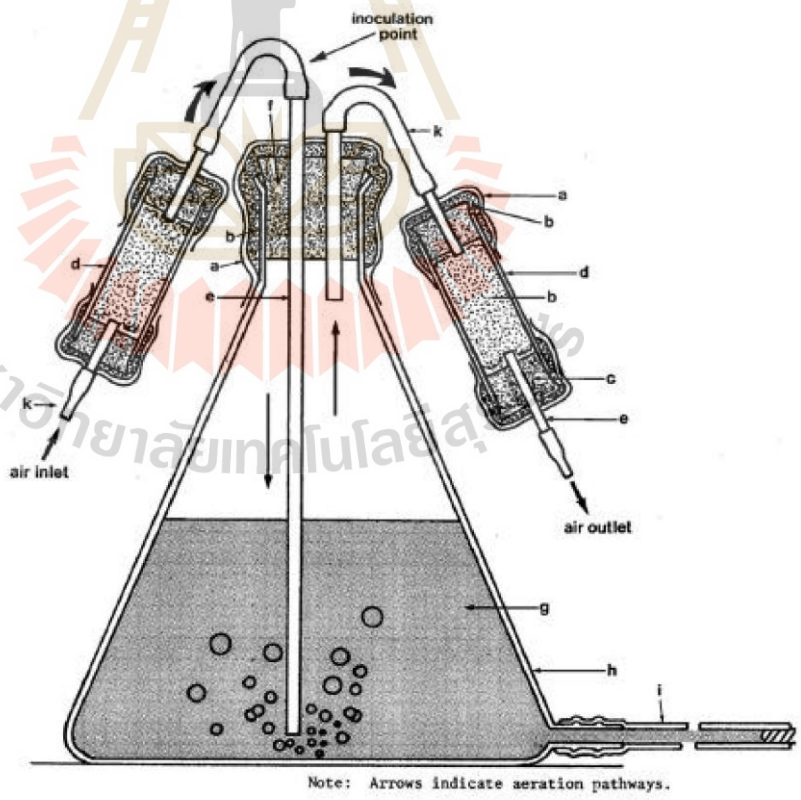

Figure 2.6 Simple glass fermentor for produce rhizobium inoculant (Somasegaran, 1985).

packed filters to prevent the entry contaminant via the airlines, seal with parafilm in the mouth flask, air inlet, air outlet and connect with an aquarium pump. Until the culture to 7 days, the rhizobium growth should determine the contamination with pH test, Gram stain, peptone glucose test and others methods.

Rhizobium inoculant uses a large scale for commercial. Mass culture requires large capacity fermenters for production. Fermentors must be simple to easily sterilize the growth medium, provide access for inoculating, sampling, aerating, and cleaning. The fermentor should be made of stainless steel for strength and corrosion resistance. The start culture was prepared starter 1-2 liter in a glass fermenter contain YEM and inoculated *B. diazoefficiens* USDA110 50 ml of starter in the flask to culture 7 days. The YEB medium was prepared in fermentor containing 100 liters and adjusted pH 6.8. The fermentor has opened all valves except the air outlet valve. The medium sterilization has operated a pressure of 15 lb/in2 at 121°C in 45 min. Further, the turner turns off and shut off cooling water. The sterile completely, the medium was determined contamination. The starter culture was inoculated in the fermentor by an aseptic technique. The fermentor has opened the airflow 3-10 liters of air per hour per liter of the medium. During the culture, the inoculant was determined the contamination and checked performed pH measurements. The contamination was detected by the gram straining. Then, the quality of inoculant was measured optical density (OD) or total plate count. Bradyrhizobium culture was usually cultured in 6-7 days (Figure 2.7).

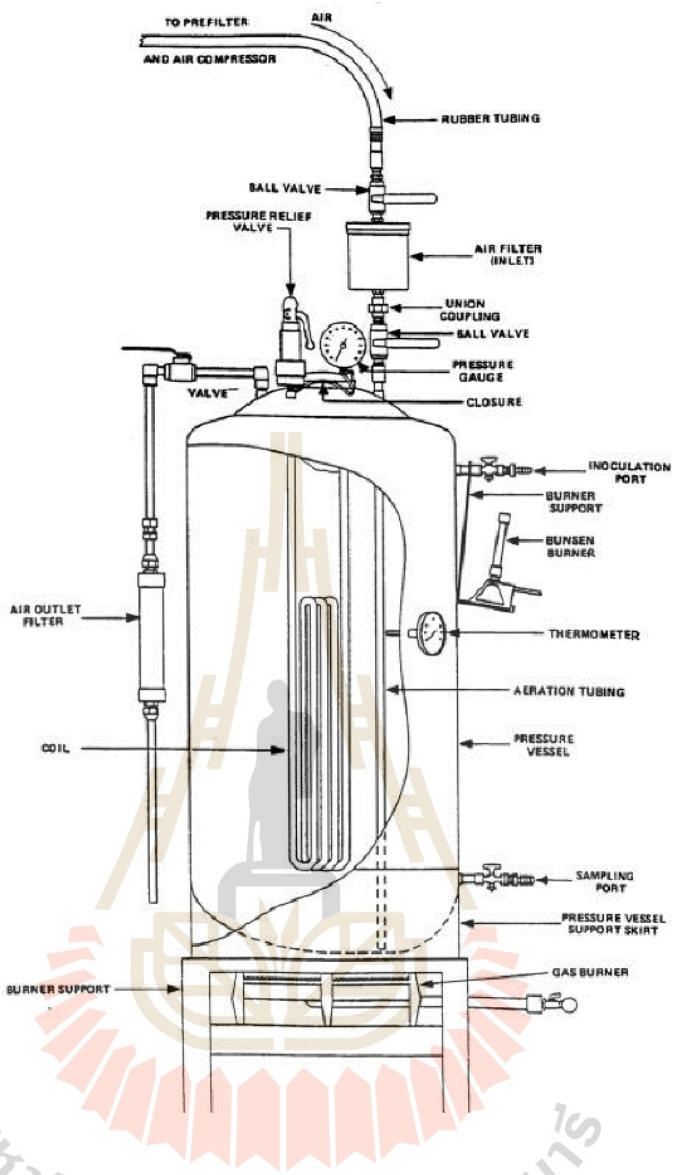

Figure 2.7 An industrial aerobic fermentor (Burton, 1984).

2.12.2 Incorporating broth culture into carriers

The large scale of commercial conditions in the United States, quality-tested broth cultures are incorporated into peat at the rate of 1 liter per kilogram of peat. After a curing period, the mixture is packaged in thin-gauge (0.05 mm) polyethylene bags (Figure 2.8). Bags of this specification permit gas exchange while minimizing moisture loss from the inoculant. The expiration date for inoculants based

on nonsterile carriers is usually 6 months. Inoculant producers in some countries, such as South Africa, Australia, and New Zealand, produce inoculants with sterilized carriers. In this case, the carrier is first packaged and then sterilized by gamma-irradiation or autoclaving. Thin-gauge (0.05 mm) polyethylene bags are used for carriers to be gamma-irradiated. Carriers to be autoclaved are packaged in polypropylene bags of the same gauge. The rhizobial broth culture is aseptically injected into the packaged carrier with a manually operated motorized syringe. Inoculants based on sterile carriers are usually of higher quality than the nonsterile carrier type. The number of viable rhizobia per gram can be between 10^9-10^{10} cells in inoculants produced with sterilized carriers. In nonsterile carriers, as in nonsterile peat, the initial number of viable rhizobia tends to be lower by at least one log after curing. The number of rhizobia added to most sterile carriers remain high during shelf life or storage because there are no other microorganisms in the carrier competing with the rhizobia. The quality of such inoculants may still be acceptable after 6-12 months, depending on the temperature during storage. Although producing inoculants based on sterile carriers is more costly than nonsterile carrier-based inoculants, mainly due to the need for sterilization facilities and labor-intensive production operations, using the dilution technique can substantially lower the production cost. Here, the broth culture is aseptically diluted with sterile water up to 1000-fold before incorporation into the sterile carrier as demonstrated in sterile peat. The low cell population in the diluted culture will multiply to the same level as with undiluted cultures during the maturing time of 5-7 days (Somasegaran, 1985; Swelim et al., 2010).

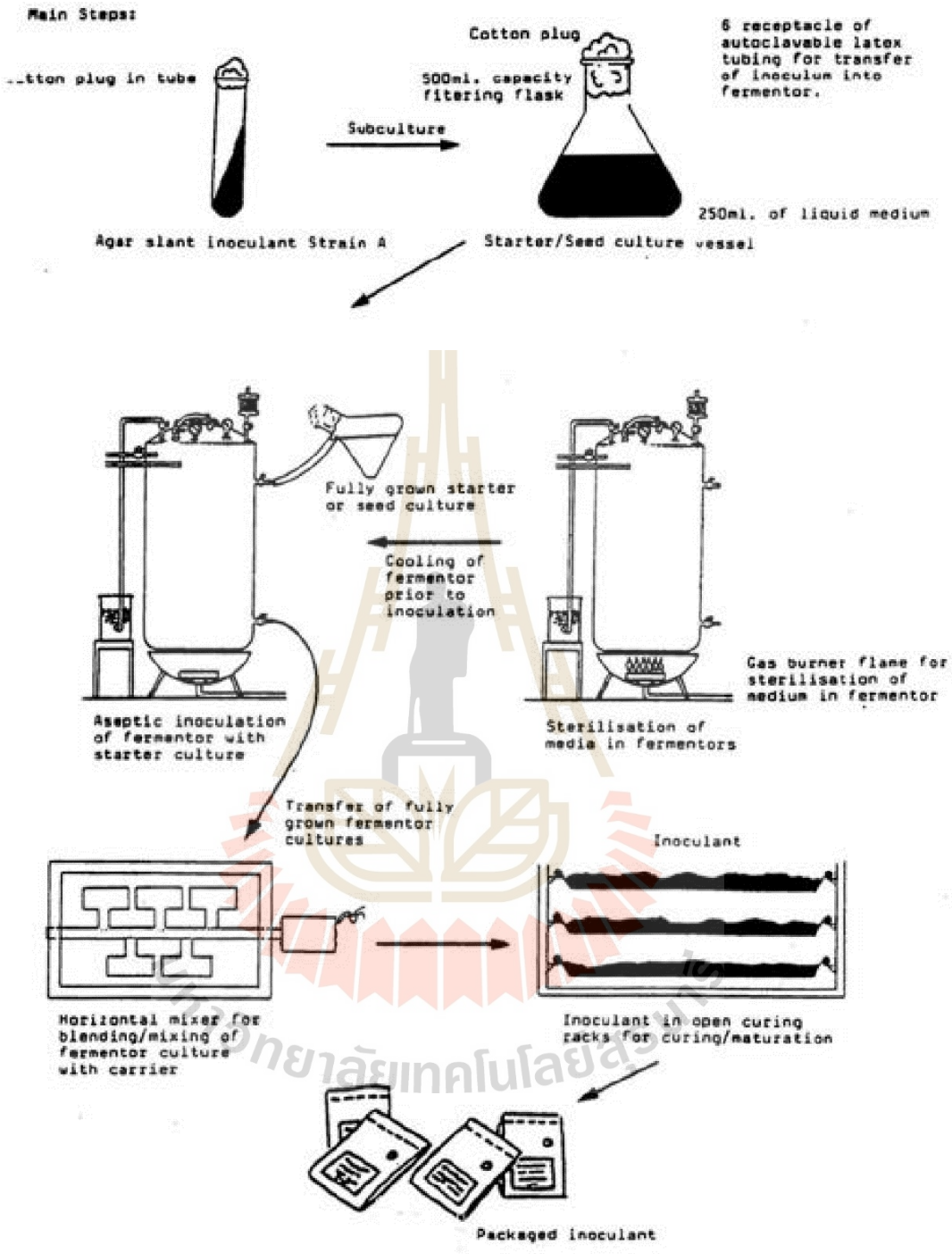

Figure 2.8 Process to inject rhizobium to inoculant carriers (Burton, 1984).

Inoculants are cured for about 2 weeks at 25-30°C to gain maximum numbers in excess of 10^8 and 10^9 cells g^{-1} for nonsterile and sterile carrier-based inoculants, respectively (Thompson, 1984). Thereafter, inoculants are stored in a refrigerated or air-conditioned environment, protected from direct light. Most inoculants are stored at 4°C and tend to survive best at this temperature. However, there are inoculant strains, including CB 627 from *Desmodium*, CB 1923 from *Centrosema*, and CB 82 from *Stylosanthes*, that have very poor survival at 4°C but have good survival at 26°C after 12 months. The final moisture content of the peat inoculant should be 40-60% on a wet-weight basis for inoculants produced with sterilized peat. A lower moisture content (30-40%) is preferred for better rhizobial survival in nonsterilized peat (Bala et al., 2011).

2.13 Development a novel technology to produce rhizobium inoculant

According to problems associated with rhizobial inoculant is a high cost to set up the laboratory and the factory. The simple technique is needed to lower the cost of inoculant production and can extend the shelf-life in the market. The inoculant production was some strategies used for improving the production, and survival of rhizobial shelf-life. In this study, since the inoculant is composed of three main components; therefore, each component could be improved as follows. Dilution technique was used for dilution of the rhizobium starter culture to produce rhizobium inoculants such as liquid-, peat- and encapsulated inoculant. Further, the lower dilution culture could grow in three carriers and be used for the pump pilot-scale of rhizobium inoculant production. The syringe- and peristaltic pump were operated the diluent of starter to injected in liquid-, peat- and encapsulated inoculant. Finally, the

inoculants were observed the shelf-life and used inoculant rhizobium to grow soybean under the field condition.

The seeds pigeonpea was infused pink pigmented facultative methylotroph (PPFM) with rhizobium inoculant 1:100, the seed was highly germinated with the seed germination (Raja at al., 2019). The injection pump has been used a dilution of 1000-folds of rhizobium culture, the starter culture was injected in peat carrier (Somasegaran, 1985).

Peristaltic-, and syringe pump were wildly used in a medical, food, laboratory, and others used. The pumps were delivered liquid, nutrients, medications, chemical from one place to another place. The pump could control the flow rate in range of 0.73 µl/h to 1500 ml/h. In addition, the pump could be controlled by manual, and program setting that advantage of using. The pumps were significantly flowed the fluids, the ability to transport fluids in low concentration, and the capability to deliver solutions at exactly set rates or computerized intervals. These issues can compromise the safe use of external infusion pumps and lead to over- or under-infusion, missed treatments, or delayed therapy (Pihl et al., 2005). The advance of syringe-, and peristaltic pump were high proficiency fluid to other containers. Therefore, this pump can be applied to dilute starters of rhizobium with distilled water to the both medium and the carriers easily. Moreover, there were many types of pumps and syringe pumps to injection the microorganism to the carries, such as the mixture was introduced in a syringe and placed on the encapsulation device to form alginate bead encapsulation.

The standard of biofertilizer production had no rule in the EU and USA, but the legal controlled in the only pest for agriculture (Malusa and Vassilev, 2014). The law standard to controlled rhizobium inoculant in Australia, the cells are concentrated

more than 10^9 CFU/g in peat and have the contamination lower than 10^6 CFU/g in peat (Herridge et al., 2002). The China legal standard for rhizobium inoculant quality, the cell is 1×10^8 CFU/g or CFU/ml (Suh et al., 2006).

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of bacterium culture and carriers

3.1.1 Microorganism

Bradyrhizobium diazoefficiens USDA110 was used throughout in this experiment. *B. diazoefficiens* strain USDA110 was obtained from Department of Agriculture, Bangkok, Thailand.

3.1.2 Preparation of liquid and solid carriers

Three types of rhizobial inoculant were prepared in this study, including liquid inoculant using YEM broth based (Vincent, 1970), solid peat-based inoculant, and alginate encapsulated inoculants.

(i) Liquid-based inoculant: The YEM liquid medium was added with 2% Polyvinylpyrrolidone (PVP) (w/v) and adjusted to pH 6.8 before packing 99 ml in 120 ml-plastic bottles (Tittabutr et al., 2007). Liquid carrier inoculant was sterilized by autoclaving at 15 lbs pressure, 121°C for 15 minutes.

(ii) Peat-based inoculant: Peat was used as carrier and its chemical and physical compositions were determined prior to use (total nitrogen 1.05%; organic matter 93.12%; total P 0.38%; total K 0.24%; available Ca 2.46%; Mg 0.24%; Fe 0.32%; Ze 0.004%; Cu 0.004%; pH 7.19; EC 0.755 ds/m; particle size 80-100 mesh). Peat was neutralized to obtain the final pH at 7.0. In the meantime, 25% YEM broth was added and packed 90 g in a low-density polypropylene bag (18×28.5 cm). Peat

carrier was sterilized by autoclaving at 15 lbs pressure (121°C) for 20 minutes for three times.

(iii) Alginate encapsulated cell-based inoculant: The solution of alginate was prepared by added 2% w/v sodium alginate and skim milk without Ca 0.75% w/v, (Shcherbakova et al., 2018) in YEM broth and adjusted the pH to 6.8. The solution was prepared in 1 l in Erlenmeyer flask and sterilized by autoclaving at15 lbs pressure (121°C) for 15 minutes.

3.1.3. Incorporating starter culture into liquid, solid carriers and encapsulated inoculant to determine the lowest cell number as starter culture for inoculant production

The culture of USDA110 was conducted by culturing in YEM broth. The bacterial culture was shaken at 200 rpm for 5 days at 30°C. The starter culture of USDA110 was manually diluted to be 10^3, 10^4, 10^5, 10^6, 10^7 and 10^8 CFU/ml using 0.85% (w/v) NaCl solution as diluent. Then, each diluted starter culture was inoculated into different types of inoculant as following;

(i) 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 (28-30°C) for 28 days without shaking.

(ii) Peat-based inoculant: 1 ml of each diluted starter culture and 9 ml sterilized 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 (28-30°C) for 28 days.

(iii) 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, 28-30°C. 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. Peat inoculant was determined by total plate count and determined most probable number (MPN) with plant (soybean: *Glycine max* (L) Merr) 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 28°C/23°C. 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 was collected as the number of plants nodulated by USDA110. 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.

3.2 Application of pumps for inoculant injection

The selected carriers and appropriate amount of starter was used for liquid, solid and polymer encapsulated inoculant. Peristaltic-, and syringe pump machines were used to make different dilutions (Figure 3.1 and Table 3.1).

(i) The liquid inoculant, the step one syringe pump (No. 1) was worked to inject bacterial starter volume as 1 ml (log phase at 10^9 CFU/ml) into 1 L of bottle containing 999 ml of normal saline to obtained the final concentration bacterial cells at 10^6 CFU/ml. The bacterial suspension at 1^{st} step was infused by syringe pump (No. 2) with operating the flow rate at 10 µl (log phase at 10^6 CFU/ml), and connected with peristaltic pump (No. 3) that flowed YEM broth as 0.999 ml in 1 L of bottle. The bacterial suspension was inoculated into 99 ml of YEM broth in 125 ml plastic bottle to obtain the final concentration bacterial cells at 10 CFU/ml. The result of initiation of cell number each bottle was determined by total plate count as mentioned above. The bottles of liquid inoculant were incubated at room temperature at 28±l°C for 1 month for counting cell number again.

(ii) The peat inoculant, the step 1^{st} syringe pump (No. 1) was operated to make dilution the bacterial starter volume at 0.1 ml (log phase at 10^9 CFU/ml) into 1 L of bottle containing 999.9 ml of sterilized normal saline to obtain the final concentration

bacterial cells as 10^5 CFU/ml. The bacterial suspension at the 1st step was flowed by syringe pump (No. 2), the rate of bacterial starter culture at 10 µl (log phase at 10^5 CFU/ml) was mixed with peristaltic pump (No. 3) that flowed distilled water at 9.999 ml containing in 1 L of the bottle. The bacterial cells culture were injected into 90 g of peat packed in a low-density polypropylene bag to obtain the final concentration of cells as 10 CFU/g. The final moisture content was adjusted to 40%. The initiation cell number in each bag was determined the cell number by total plate count as mentioned above.

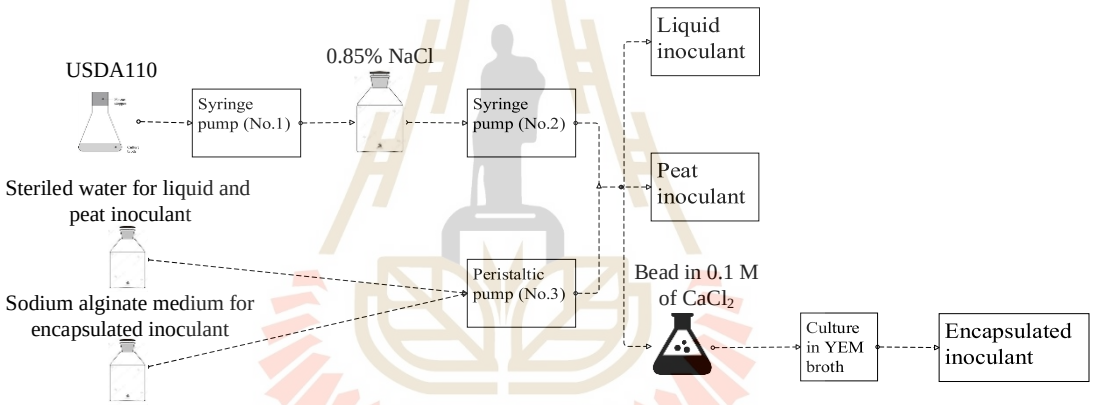

Figure 3.1 Model of inoculant production from pump by diluted starter into liquid, peat and encapsulated inoculants.

(iii) The alginate encapsulated inoculant, the step 1st syringe pump (No. 1) was used to inject bacterial starter culture at 0.1 ml (log phase at 10^9 CFU/ml) into 1 L of bottle containing 999.9 ml of sterilized normal saline to obtain the final concentration bacterial cells as 10^5 CFU/ml. Step 2: the bacterial suspension at 10 µl/min (log phase at 10^5 CFU/ml) was flowed from syringe pump (No. 2) connected with peristaltic pump (No. 3) that flowed sodium alginate solution 1 L in flask. The two solutions

were mixed well then, bacterial suspension was dropped into 0.1 M $CaCl_2$ solution contained in the 250 ml flask to obtain the final concentration of cells as 10 CFU/g. The flask was interval shaken by hands during dropping to avoid the agglomeration of beads. Macro beads were formed instantly upon contact of the droplets with the solidifying solution. The macro beads were allowed to cure in the $CaCl_2$ solution for 30 min. This procedure produced macro beads size average 3.43 mm. Each concentration of macro beads was rinsed 4 times with 0.85% NaCl before counting the cells number by total plate count. The wet beads were transferred to fresh YEM broth into culture for 7 days at room temperature with shaking 180 rpm. After that the wet beads were rinsed again prior to determine viable cells number using total plate count technique. Then, the bead was packed into a low-density polypropylene bag (Shcherbakova et al., 2018). The cell population in alginate beads inoculant was determined using total plate count in YEM Congo red the same above experiment. The number of cells was collected as 1 month, analysis the data and choose the optimum treatment to next experiment.

Table 3.1 Pumps characteristics

Type of pumps	Model No	Tubing diameter (mm)	Drive (rpm)	Inoculant types	Speed rate (ml/min)	Total volume (ml)	Copy mode (Sec)
Syringe pump No. 1	Cole Vernon Hills, Illinois 60061	3.1		Liquid	1.15	1	Stop
				Peat	0.15	0.1	Stop
				Encapsulated	0.15	0.1	Stop
Syringe pump No.2	Cole Vernon Hills, Illinois 60061	3.1		Liquid	0.015	0.01	Continuous
				Peat	0.015	0.01	Continuous
				Encapsulated	0.015	0.01	Continuous
Peristatic pump	Masterflex, USA, Model 77202-60	3.1	600	Liquid	6.59	0.999	15
				Peat	60.59	9.999	15
				Encapsulated	3	Continuous	2

3.3 Investigation of rhizobium inoculants shelf-life

The inoculants were incubated at room temperature (28-30°C) for 6 months, and the viable cells number of USDA110 from each type of inoculant was determined by total plate count (on YEM Congo red medium) and plant infection (MPN) methods as mentioned above. The data were collected for every month. Based upon the effect of the additives on shelf-life of rhizobia, the good quality of inoculant (viable cell number more than 10^6 CFU/ml (for liquid inoculant) or 10^6 CFU/g (for peat- and alginate bead-inoculants) were selected for further experiments.

3.4 FA preparation

Young male White New Zealand rabbits (6 months) were immunized with prepared USDA110 cell antigen by injection at intravenous every day for 5 days. The blood collection determined the antibody titer to reaction using antigen boiled

USDA110 culture. The optimization serum 1:1600 was used to purity by dialysis bag to obtain polyclonal antibody serum. Serum was stored at -20˚C until use. It was tested to detect rhizobia in nodules by the through FA procedure. A fluorescent antibody technique, the rhizobium sample was a thin smear and heat fix or nodule section as 45 µm. Then smear was covered with 1:1600 diluted rabbit antiserum, and incubated for 20 min. Further, the excess antiserum was temporarily rinsed with phosphate-buffered saline (PBS). The smear covered with FITC conjugate of goat anti-rabbit globulin and incubated for 20 min. After that excess FITC conjugate was rinsed with PBS for 20 min. FITC was washed with distilled water in 10 min. Finally, the sample was dried by air and detected under a fluorescent microscopy and observed confocal microscopy for nodule section (Somasegaran, 1985).

3.5 Field experiment

Field experiment of soybean (*Glycine max* (L) Merr) variety [Chiangmai 60] was performed at Nakhon Ratchasima (14˚52' 16.11"N/ 102˚ 1' 31.95" E, July-October 2020). The physio-chemical characteristics were sandy loam, pH 6.67, EC 0.025 ms/cm, OM 0.59%, available P 44.86 ppm, exchangeable K 53.53, available Ca 230 ppm). The native rhizobium persistence was not detected when using plant MPN method. Three types of inoculant from pump pilot-scale experiment as liquid inoculant, peat and alginate capsulated inoculant, and non-inoculation were used in this experiment. Each experiment was conducted in randomized complete block design (RCBD) with 3 replications. Four-row plots were used with 50 cm between rows, 20 cm between plant, 2 seeds/hole, and plots were kept 100 cm spacing between blocks. Each plot of size was conducted 2×3 m. Each of liquid, peat, and alginate capsulated

inoculant was with 1 month old inoculated (10^7 CFU/seed). Liquid inoculant was mixed with the seeds before growing under the ground. The peat inoculant was coated over the seeds in each row prior to covering the seeds with soil. The alginate capsulated was mixed with soybean seeds and cultured in hole of soil directly (Albareda et al., 2008). The soybean was harvested at 30, 45, and 65 dai after planting, to determine plant height, nodule number, nodule dry weight, shoot dry weight, root dry weight, total plant dry weight, chlorophyll content (the third and fourth leaves from the top of the plant were measured five sub-leaves by SPAD meter), N_2 fixation by Acetylene Reduction Assay (ARA), and FA from root nodule observed in florescent microscopy and confocal microscopy. The soybean yield was harvested at maturity stage at 100 days after plantation.

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

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 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, seven levels of cell concentration starting from 10 to 10^6 CFU/unit were used to produce liquid-, peat-, and encapsulated cell-based inoculants. The result showed that bradyrhizobial cells at all starting concentrations could grow well in the bottle although without shaking (Table 4.1). This probably due to in the bottles containing bacterial suspension had air space of 25 ml, the remained oxygen in the bottle might be properly supplied for bradyrhizobium growth. The bradyrhizobium could grow under microaerophilic condition at less than 0.01 atm (Shakhawat, 2007). 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 could incresed 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. diazoefficiens* 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). The supplementation of the polymer additives could support cell growth of rhizobium strain USDA 3100 as high as 10^9 CFU/ml in liquid inoculant (Mohamed et al., 2019).

Table 4.1 The liquid inoculant production of rhizobial use dilution technique in YMB.

Treatments	Log10 number of cells growth CFU/ml (days)				
	0	**7**	**14**	**21**	**28**
NI	-	-	-	-	-
10	0.20±0.17^{f}	7.64±0.03^{c}	8.58±0.09	8.48±0.12	8.78±0.12
10^2	2.23±0.05^{e}	7.76±0.02c	8.70±0.17	8.20±0.35	8.88±0.12
10^3	3.21±0.03^{d}	7.99±0.00^{b}	8.50±0.10	8.29±0.22	8.75±0.25
10^4	4.09±0.02^{c}	8.00±0.04^{b}	8.57±0.10	8.37±0.02	8.64±0.09
10^5	5.23±0.04^{b}	8.05±0.12^{ab}	8.53±0.04	8.30±0.23	8.70±0.11
10^6	6.23±0.02^{a}	8.17±0.12^{a}	8.45±0.19	8.53±0.05	8.68±0.16

Mean followed by the different letters that mean was significantly different from the treatments in the column. Significance at $p \leq 0.05$ is demonstrated by mean standard error bars (n = 3).

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 conditions 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 when determined by total plate count (Table 4.2).

Table 4.2 Cells growth of *B. diazoefficiens* strain USDA110 in peat inoculant determined by total plate count.

Treatments	Log10 number of cells growth CFU/g (days)				
	0	**7**	**14**	**21**	**28**
NI	-	-	-	-	-
10	0.16±0.28^{f}	6.93±0.43^{bc}	6.70±0.05^{c}	8.43±0.25	8.38±0.27
10^2	2.27±0.20^{e}	6.60±0.35^{c}	7.18±0.46^{b}	8.31±0.15	8.33±0.13
10^3	3.21±0.05^{d}	7.43±0.53^{ab}	7.29±0.07^{b}	8.35±0.12	8.41±0.13
10^4	4.24±0.04^{c}	7.81±0.15^{a}	7.39±0.12^{b}	8.39±0.08	8.39±0.02
10^5	5.22±0.04^{b}	7.53±0.02^{a}	7.50±0.13^{ab}	8.32±0.19	8.21±0.19
10^6	6.18±0.07^{a}	7.99±0.14^{a}	7.86±0.22^{a}	8.60±0.09	8.42±0.09

Mean followed by the different letters that mean was significantly different from the treatments in the column. Significance at $p \leq 0.05$ is demonstrated by mean standard error bars (n = 3).

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. In the case of the MPN method, the cell concentration in peat carrier was significantly reduced the when dilution was made manually (Table 4.3). Although the all cell starter culture was increased the cells containing 10^7 CFU/g in peat at 7 dai. Furthermore, the cells number was evaluated as 10^8 CFU/g at 21, and 28 dia. The cell grown in peat inoculant was not different in both high or low cell starters. However, the cell number in total plate count was higher than the MPN at 1 and 2 weeks. The cells were reached up to 10^8 CFU/g after 3 and 4

weeks with the similar cell number in two methods. 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 at 10^6, 10^7, or 10^8 cells/ml that were injected into sterile peat, and the cells could grow as 10^9 CFU/g at 7 dai (Somasegaran, 1985).

Table 4.3 Cells growth of *B. diazoefficiens* strain USDA110 in peat inoculant determined by MPN method.

Treatments	**Log10 number of cells growth CFU/g (days)**				
	0	**7**	**14**	**21**	**28**
NI	-	-	-	-	-
10	1.56±0.58d	7.56±0.58	8.57±0.59	9.26±0.00	8.57±0.59
10^2	2.56±0.58c	7.56±0.58	8.92±0.59	8.92±0.59ab	8.57±0.59
10^3	3.90±1.15c	7.56±0.58	8.75±0.52	8.57±0.59ab	8.92±0.59
10^4	5.23±0.00b	7.90±0.58	8.57±0.59	8.23±0.00b	8.23±0.00
10^5	5.90±0.58ab	7.56±0.58	8.92±0.59	8.57±0.59ab	8.23±0.00
10^6	6.90±0.58a	7.74±0.50	9.45±0.33	8.92±0.59ab	8.57±0.59

Mean followed by the different letters that mean was significantly different from the treatments in the column. Significance at $p \leq 0.05$ is demonstrated by mean standard error bars (n = 3).

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 forming 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 cells when using high concentration of starter at 10^3 to 10^6 CFU/g were significantly higher than those of using low concentration of starter culture at 10 and 10^2 CFU/g (Table 4.4). 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* with starter culture at 10^{11} CFU/g in beads was grown in fresh TYG medium and then the cell could increase up to 10^{12} CFU/g (Bashan et al., 2002). The advantages of using alginate microspheres were able to controlled the release of bacteria, slow biodegradation, and extension of 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.

Table 4.4 Cells growth of *B. diazoefficiens* strain USDA110 in the alginate bead inoculant production.

Treatments	Log10 number of cells growth CFU/g (Times days)				
	0	**7**	**14**	**21**	**28**
NI	-	-	-	-	-
10	1.86±0.28^{f}	6.23±0.05^{c}	8.98±0.08^{c}	9.47±0.03^{ab}	9.58±0.14^{ab}
10^2	2.61±0.19^{e}	6.63±0.41^{b}	9.12±0.19^{bc}	9.56±0.13^{ab}	9.57±0.18^{ab}
10^3	3.86±0.04^{d}	7.64±0.14^{a}	9.16±0.10^{bc}	9.34±0.10^{b}	9.55±0.12^{b}
10^4	4.83±0.07^{c}	7.73±0.26^{a}	9.33±0.12^{ab}	9.56±0.25^{ab}	9.86±0.14^{a}
10^5	5.32±0.06^{b}	7.84±0.04^{a}	9.39±0.03^{ab}	9.56±0.11^{ab}	9.79±0.06^{ab}
10^6	6.08±0.10^{a}	7.89±0.04^{a}	9.53±0.12^{a}	9.60±0.11^{a}	9.83±0.11^{a}

Mean followed by the different letters that mean was significantly different from the treatments in the column. Significance at $p \leq 0.05$ is demonstrated by mean standard error bars (n = 3).

4.2 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 4.1 and 4.2).

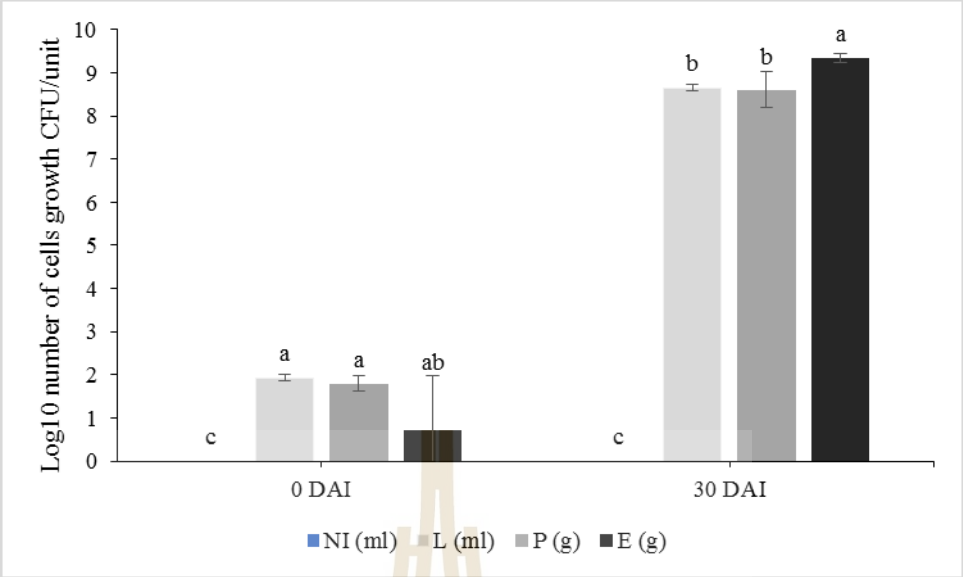

Figure 4.1 *B. diazoefficiens* USDA110 inoculant production using the syringe- ad peristaltic-pumps, (a) inoculant starter, (b) inoculant productions at 30 DAI (liquid inoculant (L), peat inoculant (P) and bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

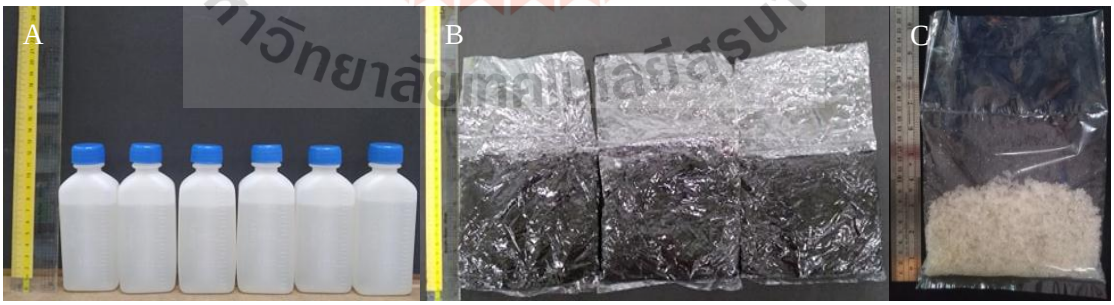

Figure 4.2 The *B. diazoefficiens* USDA110 inoculant was produced by pump pilot-scale (liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)).

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 to10^7 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 86.6, 66.6, and 50 CFU/unit in liquid-, peat-, and alginate encapsulated cell-based inoculants, respectively. Then, the concentration of cell was 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.

4.3 Shelf-life of *B. diazoefficiens* USDA110 in various types of inoculants

The survival of USDA110 in each type of inoculant was demonstrated in Figures 4.3 and 4.4. 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 and total plate count.

The lowest cells survival was found in liquid-based inoculant when analyzed using MPN technique (Figure 4.3) and total plate count (Figure 4.4). Although the liquid inoculant containing PVP, it could protect cell survival at 10^8 CFU/ml for 90 dai when stored at room temperature. Moreover, the cell number in liquid inoculant was still increased at 10^9 CFU/ml for 120 dai. Then, cell survival in liquid inoculant was slowly decreased at 10^8 CFU/ml for 150 dai, besides cell growth was slightly increased up at 10^9 CFU/ml again at 180 dai. The cell number was highly increased in the liquid inoculant at 30 dai when total plate count was employed. The survival of cell in liquid inoculant was slightly reduced from 10^8 to 10^7 CFU/ml at 60 dai. Although, the liquid inoculant was maintained the shelf-life of USDA110 10^8 CFU/ml at 90 to 180 dai. However, liquid inoculant is easy to prepare and contain high nutrient (Albareda et al., 2008). The *B. diazoefficiens* in G6+PVP medium could maintain 10^9 CFU/ml period storage at 25˚C for 180 dai (Singleton et al., 2002), and the *B.*

diazoefficiens USDA3100 was cultured in YEM-broth+2% PVP that prolonged shelf-life as 10^8 CFU/ml during storage at 25-35˚C for 60 dai (Mohamed. S. S. et al., 2019).

In peat-based inoculant, cells were grown up to 10^8 CFU/g as enumerated by MPN technique 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, 120, 150, and 180 DAI (Figure 4.3). Another dada, the survival of bradyrhizobium in peat inoculant was maintained at 10^8 CFU/g when determined by total plate count at 30 dai (Figure 4.4). Furthermore, cell number was slightly decreased at 10^7 after storage at 60 dai. Although, the survival number was again increased at 10^8 CFU/g until 150 dai. However, the shelf-life number was regressively reduced at 10^7 CFU/g in the last month at 180 dai. Based on the results, the data obtained from MPN and total plate count were correlated. 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). Peat has been postulated to contain small nutrient-supplemented of pumice that supports bacteria growth (Einarsson, Gudmundsson, Sverrisson, Kristjansson, & Runolfsson, 1993). Moreover, rhizobial cell could be immobilized in peat carrier and the low moisture content in peat affected cells survival (Žvagiņa, Petriņa, Nikolajeva, & Lielpētere, 2015). The suitable moisture content in peat for maintaining the shelf-life of bradyrhizobium is in the range of 30-50%, and when the moisture was increased up to 50%, fungi contamination was observed (Temprano et al., 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 & 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).

Interestingly, the number of survival cell in alginate encapsulated cell-based inoculant was higher than 10^9 CFU/g at 120 dai storage (Figure 4.3). The encapsulated inoculant was slightly decrease as 10^8 CFU/g when stored at 150 and 180 dai. The survival of the cell at 10^9 CFU/g was extremely preserved in encapsulated based inoculant higher than those of peat- and liquid inoculants after storage at 30 dai (Figure 4.4). Then, the cell number was slightly decreased at 10^7 CFU/g at 60 dai. After that, the number of the cell was increased back to 10^8 CFU/g during 120 to 180 dai. Sodium alginate was the most accepted hydro-gel, low toxicity, low cost, quick gelation, and biocompatibility characteristics which is suitable for cell encapsulation (Saiprasad, 2001). However, it has been reported that the alginate granules cells of *B. diazoefficiens* M8 at initial concentration of 10^9 CFU/g were reduced at 3 months of storage (Shcherbakova et al., 2018).

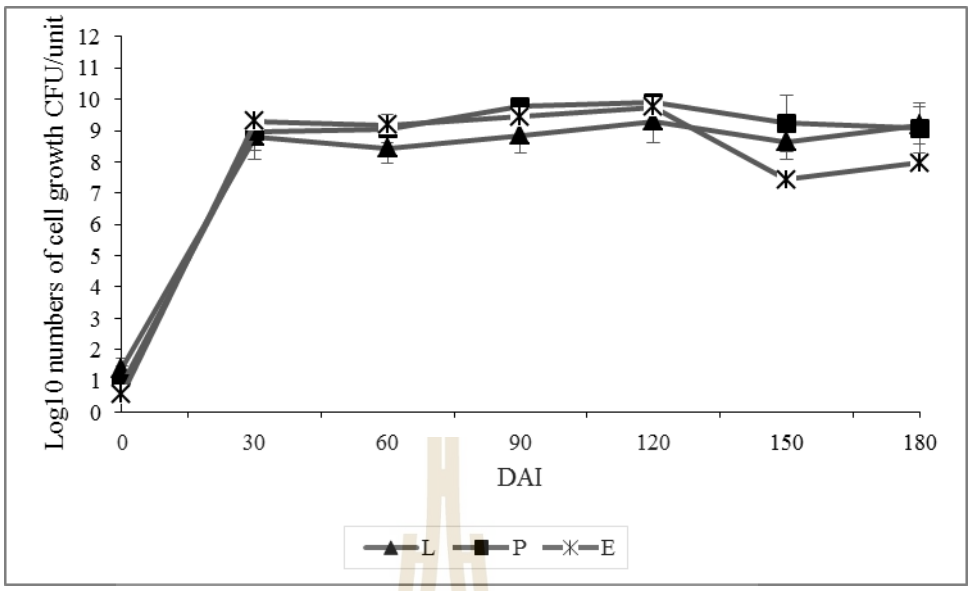

Figure 4.3 The *B. diazoefficiens* USDA110 inoculant production was determined the cell survival by MPN method (liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)); The cell survival number of *B. diazoefficiens* USDA110 inoculants in liquid (L), peat (P), and encapsulated bead (E) after storage 0, 30, 60, 90, 120, 150, and 180 dai.

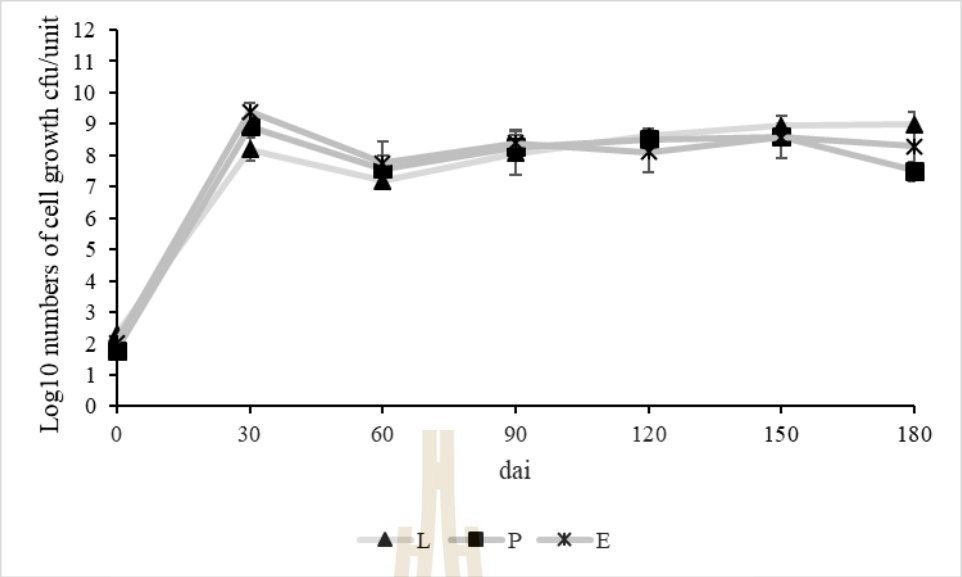

Figure 4.4 The *B. diazoefficiens* USDA110 inoculant production was determined the cell survival by total plate count (liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)); The cell survival number of *B. diazoefficiens* USDA110 inoculants in liquid (L), peat (P), and encapsulated bead (E) after storage 0, 30, 60, 90, 120, 150, and 180 dai.

4.4 Effect of inoculant production on soybean under field condition

The three types of *B. diazoefficiens* USDA110 inoculant produced from peristaltic-, and syringe-pump by low concentration of starter culture were subjected to test their symbiosis performance with soybean in the field condition (Figure 4.5). Soybeans height were significantly different when compared with non-inoculated treatment, the soybeans height was 32.97, 39.57, 40.55, and 39.93 cm at 30 dai in non-inoculated plant, plant inoculated with liquid-, peat-, and alginate encapsulated-inculants, respectively. However, the plant height of soybean was not significantly different among the three types of inoculant in liquid-, peat- and encapsulated

inoculant at 45 dai, but significantly different from non-inoculated plant. The average plant height was 47.67, 61.33, 67.33, and 63 cm in non-inoculated plant, plant inoculated with liquid, peat, and alginate encapsulated inoculants, respectively. Finally, soybean height was not significant in liquid-, peat-, and encapsulated inoculant, the averages were 72, 76.33, and 71.4 cm at 65 dai, respectively. Moreover, the plant height was higher than non-inoculated plant 16, 20.33, and 15.4 cm in liquid-, peat-, and encapsulated-inoculants, respectively. It has been reported that adding N fertilizer at 50 kg/ha^{-} with rhizobium inoculant could increase plant height equivalent to inoculate plant only with rhizobium inoculant (Ntambo et al., 2017).

Chlorophyll content in soybean was measured using SPAD unit that was significantly reduced in non-inoculated plant (Figure 4.6), while there was no significant difference among plant inoculated with liquid-, peat-, and encapsulated inoculants at 30 dai. The highest SPAD value of 33.51 was found in plant inoculated with liquid inoculant, and the lowest SPAD value of 28.07 was found in non-inoculated plant. At 45 dai, the highest of SPAD value was found in plant inoculated with peat at 42.13 and the lowest value of 29.39 was also found in non-inoculated plant. However, there was no difference of SPAD value among plant inoculated with three inoculants of liquid-, peat-, and encapsulated inoculant. Then, the chlorophyll content was increased to 34.2, 47.89, 48.41, and 47.52 in SPAD value in non-inoculated plant, plant inoculated with liquid-, peat-, and encapsulated inoculant at 65 dai, respectively.

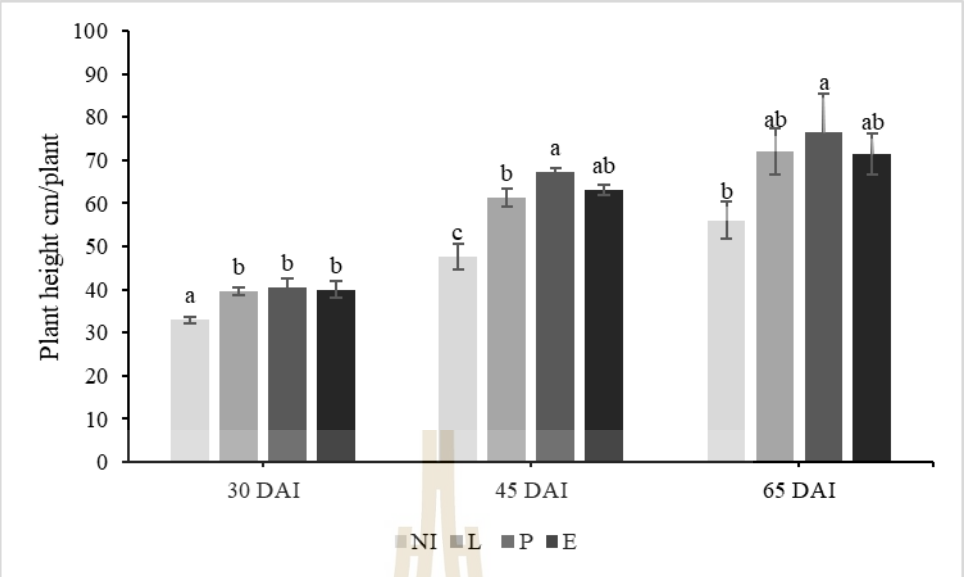

Figure 4.5 Soybean height under field condition. Soybean was inoculated with different types of *B. diazoefficiens* inoculants and data were collected at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

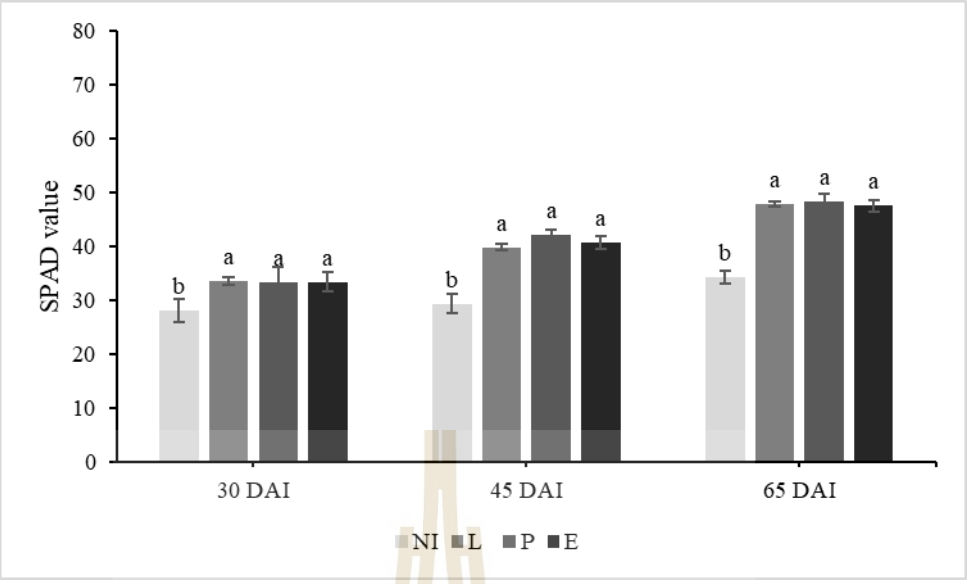

Figure 4.6 Soybean chlorophyll content under field condition was measured by SPAD reading at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

Nodule numbers per plant were significantly different as 10.75, 30, and 12.75 numbers/plant in liquid-, peat, and encapsulated inoculant at 30 dai (Figure 4.7), respectively, This result indicates the efficiency of peat-based inoculant that promote high effective nodulation in soybean (Albareda et al., 2008). Similarly, the nodule numbers were significantly dissimilar 60.33, 75, and 63,33 numbers/plant in liquid-, peat- and encapsulated inoculant, respectively at 45 dai. The data collection at 65 dai showed that the soybean nodule numbers were not different in three treatments inoculated with *B. diazoefficiens* USDA110 in liquid-, peat- and encapsulated inoculants.

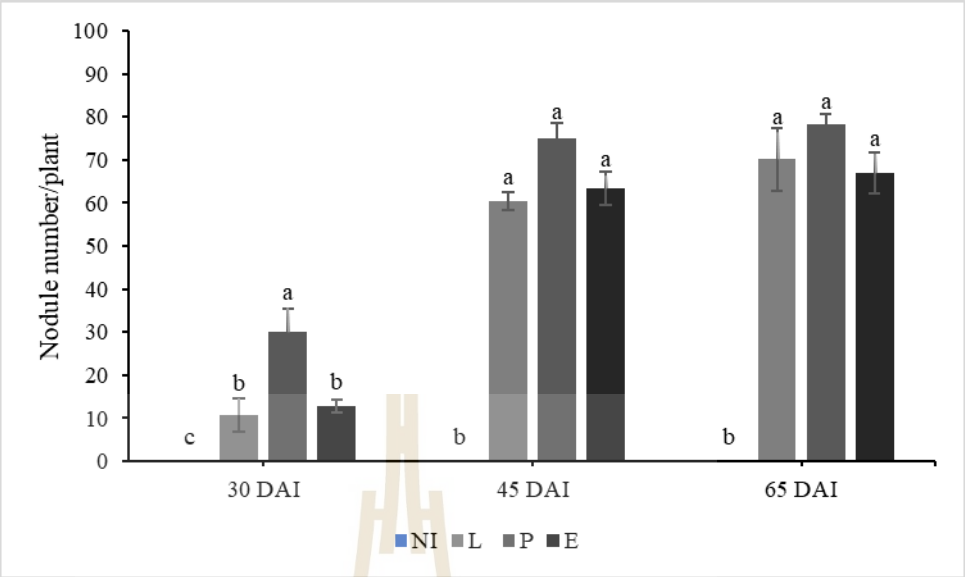

Figure 4.7 Soybean nodule formation under field condition was inoculated by non-inoculate, liquid-, peat-, and encapsulated inoculant at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

The nodules dry weight was highly increased in all inoculants from 30 dai to 45 dai (Figure 4.8). The result of nodule dry weight at 30 dai showed 0.07, 0.12, and 0.05 g/plant in liquid-, peat-, and encapsulated inoculants, respectively. The highest nodule dry weight was found in plant inoculated with peat inoculant which related to the nodule numbers. The nodule dry weight of soybean was 0.22, and 0.12 g between peat-, and liquid inoculants (Albareda et al., 2008). Then, the nodule dry weight was increased at 30, and 45 dai. The nodule dry weight was 0.37, 0.55, and 0.34 g/plant in liquid-, peat-, and encapsulated inoculant at 45 dai, respectively. Finally, the nodule

dry weight was 0, 0.7, 0.63, and 0.49 g/pant in non-inoculated plant, plant inoculated with liquid-, peat-, and encapsulated inoculants at 65 dai (Figure 4.8), respectively. The rhizobium inoculant was added 50 kg/ha of N fertilizer under the field and increased nodule dry weight higher than single rhizobium inoculant (Ntambo et al., 2017).

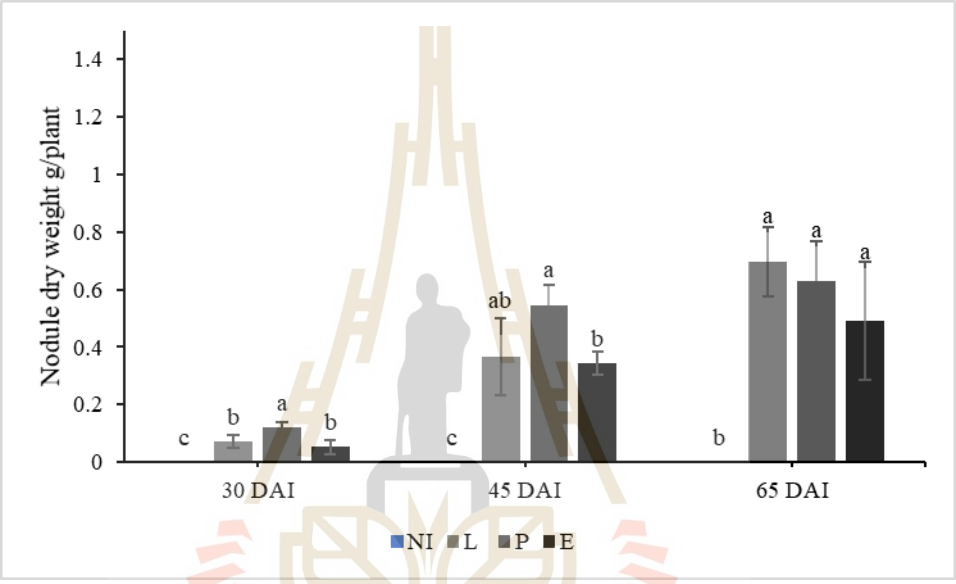

Figure 4.8 Nodule dry weight under field condition was inoculated by non-inoculate, liquid-, peat-, and encapsulated inoculant at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

Shoot dry weight biomass was significantly different showed 0.8, 1.92, 2.54, and 2.08 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant at 30 dai (Figure 4.9), respectively. Moreover, the shoot dry weight was not significant in three

types of inoculant, which higher than non-inoculated treatment at 45 dai. The high shoot biomass was peat inoculant at 7.72 g/plant, followed was encapsulated inoculant at 7.05 g/plant, and the last was liquid inoculant at 6.5 g/plant. Supplementary, the shoot dry weight was positively correlate with non-inoculant at 4.21, 11.56, 11.93, and 11.13 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant at 65 dai, respectively.

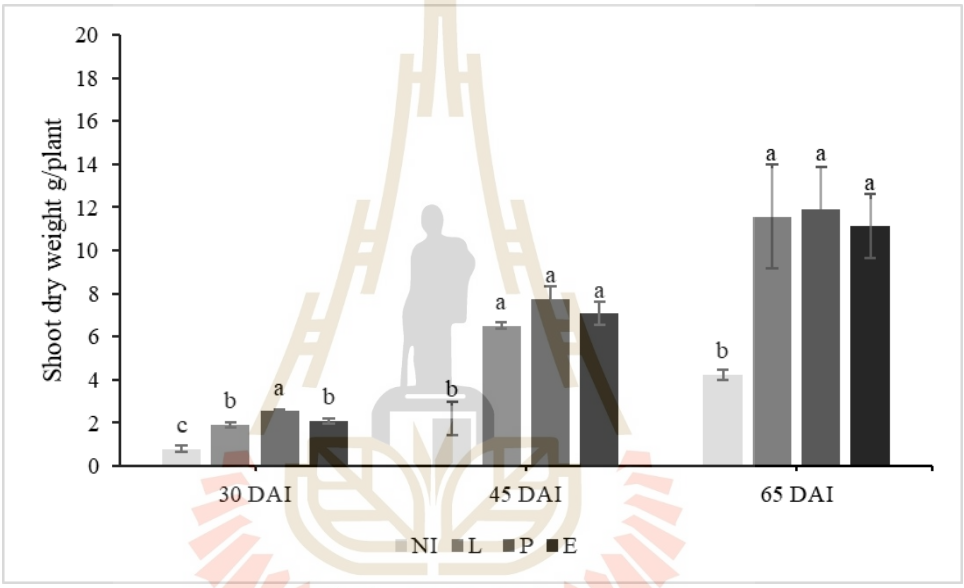

Figure 4.9 Shoot dry weight of soybean under field condition with non-inoculate, liquid-, peat-, and encapsulated inoculant at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

Root dry weight was not significantly different in three types of inoculant at 30 dai (Figure 4.10). The root dry weight was 0.19, 0.38, 0.45, and 0.34 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant, respectively. The highest root dry weight of 1.08 g/plant was recorded in plant inoculated with peat, followed by with the encapsulated inoculant at 0.98 g/plant, and with liquid inoculant at 0.98 g/plant. The lowest root dry weight was 0.37 g/plant in non-inoculated plant at 45 dai. Finally, root biomass was 0.75, 1.42, 1.36, and 1.37 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant at 65 dai, respectively. The root dry weight of liquid-, peat-, and encapsulated inoculant was 1.89, 1.81, and 1.82-folds when compared with non-inoculated plant, respectively.

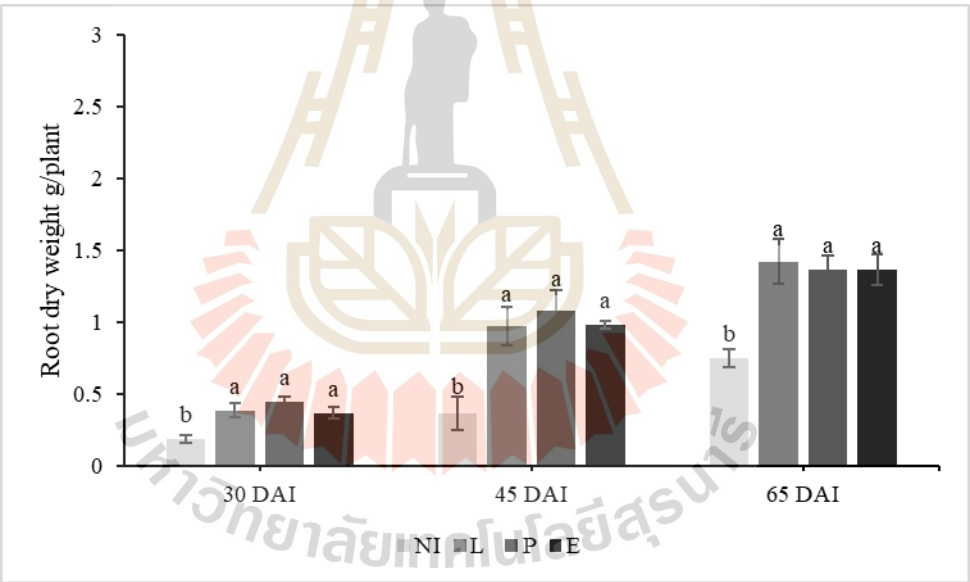

Figure 4.10 Root dry weight was measured soybean nodule under field condition with non-inoculate, liquid-, peat-, and encapsulated inoculant at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

Pod number was a significantly different at (P<0.05) that was 10.53, 45.86, 44.38, and 40.06 number/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant at 65 dai, respectively (Figure 4.11). The pod number has been increased in liquid inoculant (4.35-folds), peat inoculant (4.21-folds), and encapsulated inoculant (3.8-folds) relative to control treatment. Pod dry weight was interestingly indicated different between non-inoculant, and inoculated treatments that were 1.72, 6.66, 7.71, and 6.42 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant at 65 dai, respectively.

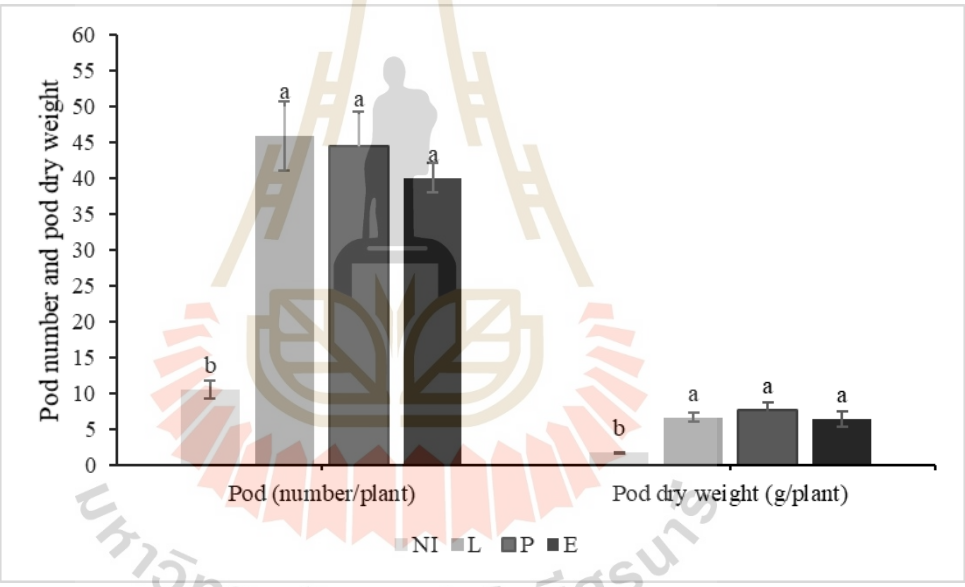

Figure 4.11 Pod number and pod dry weight of soybean under field condition when inoculated with liquid-, peat-, and encapsulated inoculant at 65 dai compared with non-inoculated plant (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

Total plant dry weight was indicated at 0.99, 2.38, 3.11, and 2,5 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant at 30 dai (Figure 4.12), respectively. The biomass of inoculated plants was 2-3 folds higher than non-inoculated plant. The total plant dry weight was 2.56, 7.85, 9.35, and 8.38 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant at 45 dai, respectively. The total dry weight has been effectively increased at 65 dai, as 6.68, 20.33, 21.63, and 19.41 g/plant in non-inoculated, liquid-, peat-, and encapsulated inoculant, respectively.

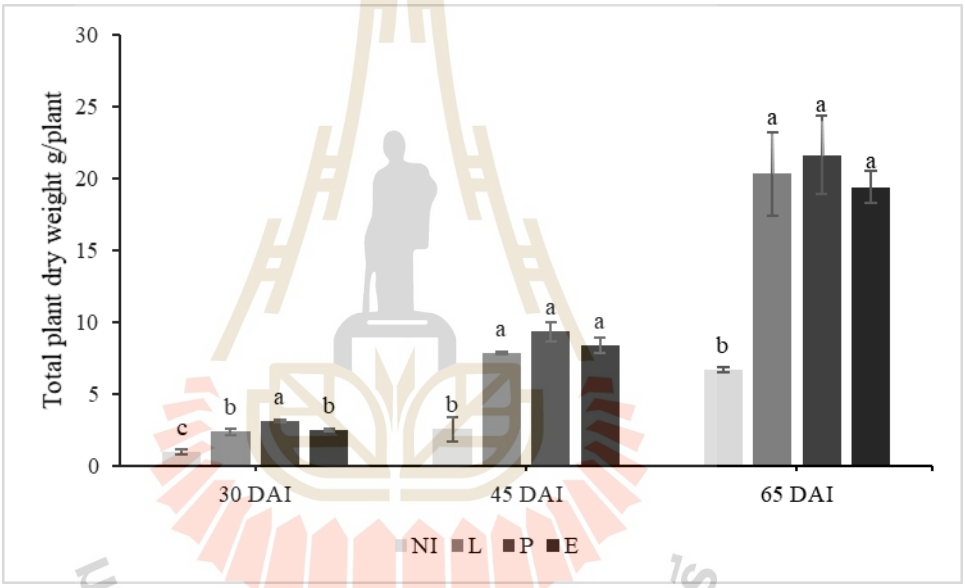

Figure 4.12 Total plant biomass of soybean under field condition with non-inoculate, liquid-, peat-, and encapsulated inoculant at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

The acetylene reduction assay (ARA) activity was measured in soybean (Figure 4.13). The result of nitrogenase activity was 10.95, 14.86, and 8.22 µmol C_2H_4/h/plant in plant inoculated with liquid-, peat-, and encapsulated inoculant at 30 dai, respectively. Although the ARA activity was not different in three types of inoculant at 45 dai, the highest and lowest ARA was found in plant inoculated with peat and liquid inoculant which are 8.38 and 6 µmol C_2H_4/h/plant, respectively. At 65 dai, the nitrogenase activity was 3.35, 4.58, and 4.58 µmol C_2H_4/h/plant in liquid-, peat-, and encapsulated inoculant, respectively. The result showed that the nitrogen fixation was highest at 45 dai and then trend to decrease at 65 dai in all inoculants.

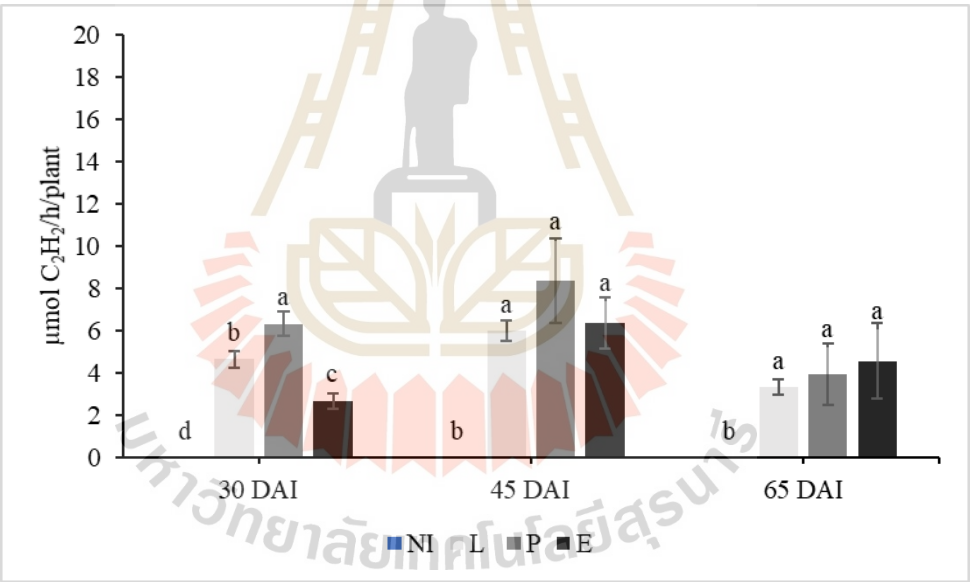

Figure 4.13 Acetylene reduction assay (ARA) was measured in soybean under field condition with non-inoculate, liquid-, peat-, and encapsulated inoculant at 30, 45, and 65 dai (non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E)). Mean followed by the different letters that mean was significantly different from the

treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

The data of nitrogen content in soybean grown under field experiment were shown in Table 4.5. Plant dry weight of inoculated soybeans was 2.9-3.23-folds higher than that of non-inoculated treatment. Percent nitrogen was contained at 1.64, 3.25, 3.23, and 3.35% in non-inoculated, liquid-, peat-, and encapsulated inoculant, respectively. The highest nitrogen content was found in soybean inoculated with encapsulated inoculant at 3.35%, and the lowest nitrogen content was non-inoculant at 1.63% at 55 dai. However, there were no significant difference among three inoculant types. The total nitrogen obtains per area was 21.91, 132.59, 139.10, and 130.07 kg/ha in non-inoculated, liquid-, peat-, and encapsulated inoculant, respectively. The total N content in inoculated plant was 5.92-6.34-folds higher than that of non-inoculated plant. Interestingly, the inoculated treatment was increased effective N-fixed at 110.68, 117.20, and 108.16 kg/ha at 55 dai in liquid-, peat-, and encapsulated inoculant, respectively.

Table 4.5 Percent of nitrogen content in soybean inoculated with Bradyrhizobium inoculant at 55 dai.

Treatments	plant dry weight (g)	% N	Total N (kg/ha)	N-fixed (kg/ha)
NI	6.68[b]	1.64[b]	21.91[b]	0.00[b]
L	20.34[a]	3.25[a]	132.59[a]	110.68[a]
P	21.63[a]	3.23[a]	139.10[a]	117.20[a]
E	19.41[a]	3.35[a]	130.07[a]	108.16[a]

Treatment symbol was non-inoculated (NI), liquid inoculant (L), peat inoculant (P), and encapsulated bead inoculant (E). Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

The yield of soybean under the field condition was higher in plant inoculated with bradyrhizobuim inoculant in all forms of liquid, peat, and encapsulated inoculants than non-inoculated plant at 100 dai (Figure 4.14). Soybean yield production was 0.99, 2.81, 3.48, and 2.56 ton/ha in non-inoculated, liquid-, peat-, and encapsulated inoculant, respectively. The total seed yield was 2.58-3.51-folds higher than non-inoculated treatment. Several researchers have indicated the liquid inoculant promoted seed yield at similarity with peat inoculant (Albareda et al., 2008; Hynes et al., 2001; Singleton et al., 2002; Tittabutr et al., 2007). However, some studies showed less effective of liquid formulations when comparison with peat carriers (Clayton et al., 2004; Rice et al., 2000). Inoculants of rhizobium did not always promote effective response with all site of soil type (Date, 2000). Adding appropriative nitrogen fertilizer was co-operated with rhizobium USDA110 that was an increased yield of soybean (Ntambo et al., 2017; Ulzen et al., 2016).

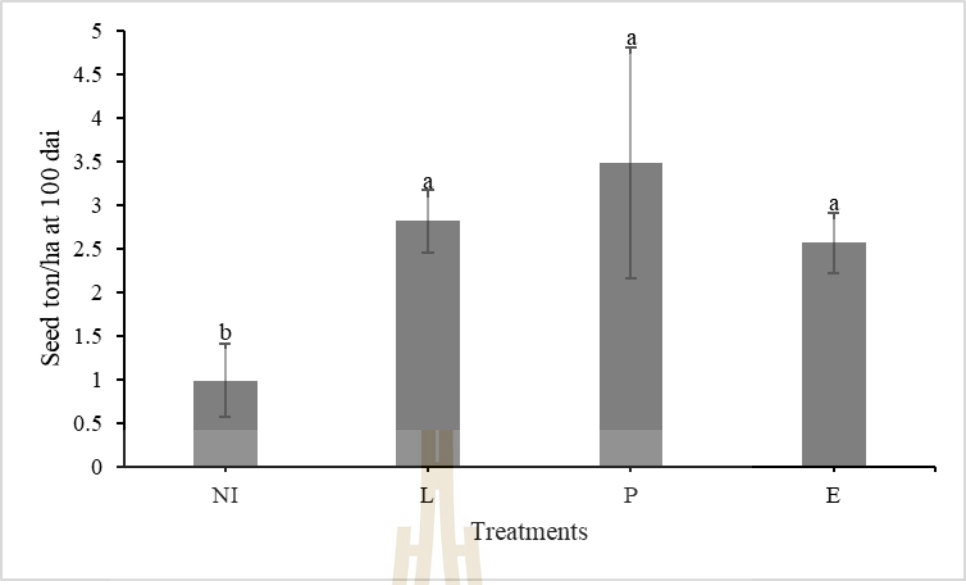

Figure 4.14 The seed of soybean when inoculated with non-inoculated (NI), liquid- (L), peat- (P), and encapsulated inoculant (E) at 100 dai under field condition. Mean followed by the different letters that mean was significantly different from the treatments in the bar graft. Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

The pictures of soybean production under the field condition were shown in Figure 4.15. The nodule sections showed red color in all treatments of liquid-, peat-, and encapsulated inoculant. The results indicate that inoculants were effectively promoted soybean growth, and yield production 4.16. Further, the inoculated bradyrhizobia were monitored by FA technique under fluorescent microscope that were indicated green in the cells of bacteroid (Figure 4.17). Interestingly, the nodule of three inoculants were greenly stained with polyclonal antibody pass antirabbit tag with FITC, which showed green color under confocal microscope (Figure 4.18). Although the polyclonal antibody from rabbit can cross with some other Bradyrhizobium (Vu et al., 2017), we may conclude that this

bacteroid belong to USDA110 since less indigenous bradyrhizobia was observed in this field due to no nodule was obtained in non-inoculated soybean.

Figure 4.15 Soybean was inoculated by different types of inoculant *B. diazoefficiens* USDA110 in non-inoculated (NI), liquid (L), peat (P), and encapsulated (E) inoculant under field condition at 55 dai; ((a) non-inoculated, (b) liquid inoculant, (b) peat inoculant, (c) encapsulated inoculant, (e) comparison in four treatments).

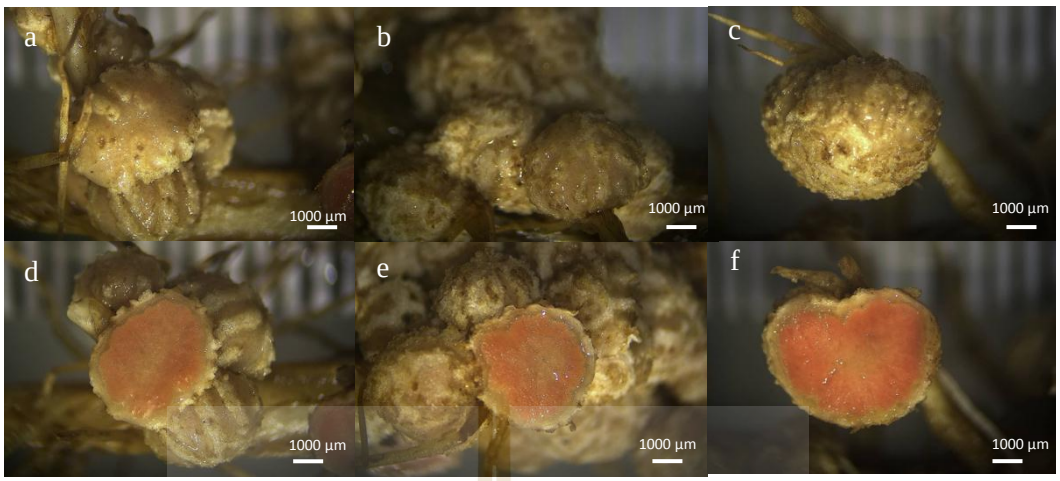

Figure 4.16 Nodules formation of *B. diazoefficiens* USDA110 when inoculated with liquid-, peat, and encapsulated inoculant at 30 dai ((a) liquid inoculant, (b) peat inoculant, and (c) encapsulated inoculant). The magnification is shown in panels a, b, and c (bars = 1000 µm). Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

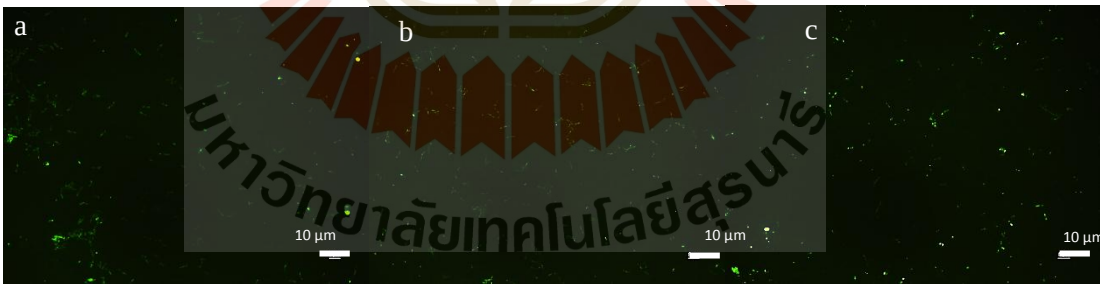

Figure 4.17 Bacteroid of *B. diazoefficiens* USDA110 was stained with polyclonal antibody and observed under florescent microscope at 30 dai ((a) liquid inoculant, (b) peat inoculant, and (c) encapsulated inoculant). The magnification is the same in panels a, b, and c (bars = 10 µm). Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

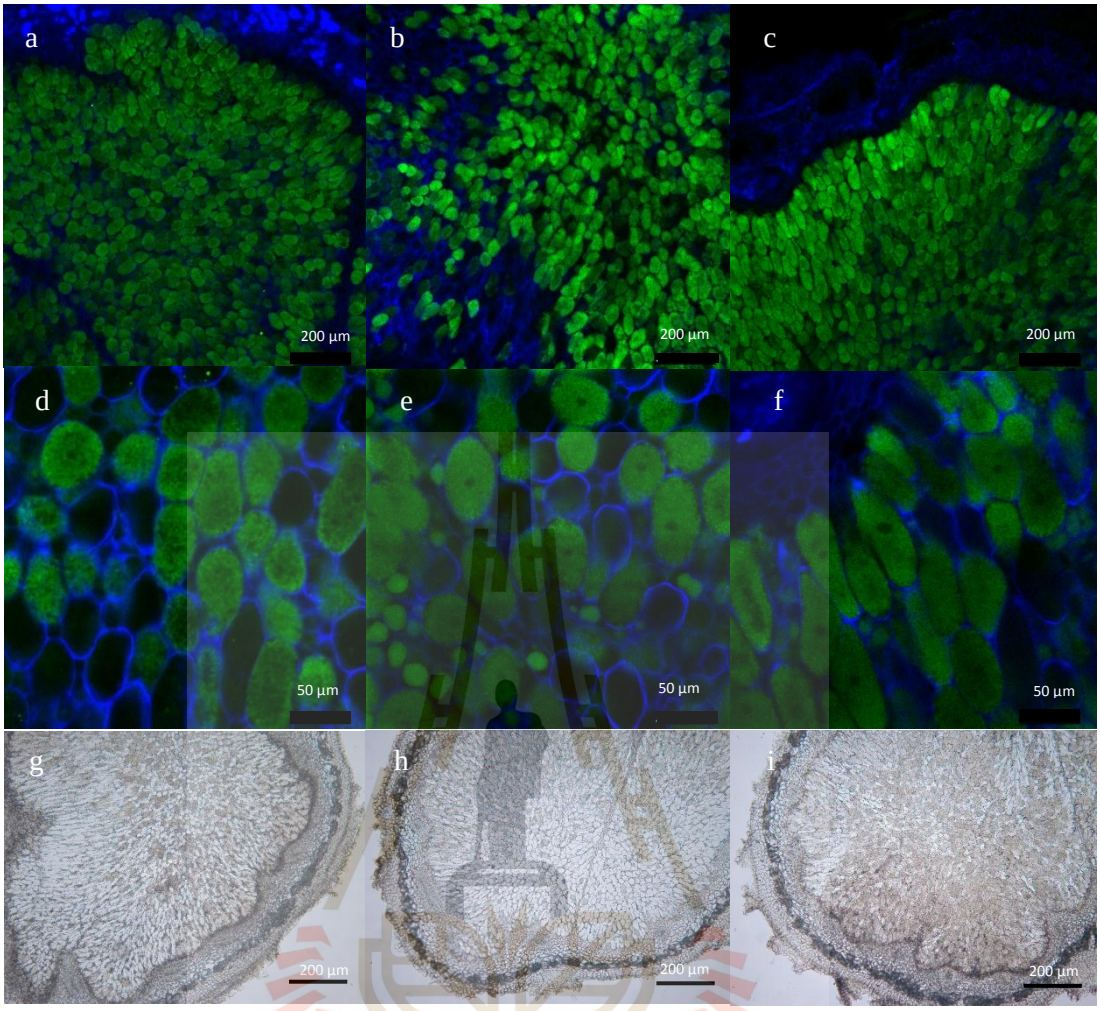

Figure 4.18 Nodule of soybean inoculation with *B. diazoefficiens* USDA110 in various types of inoculant and was stained with polyclonal antibody to observe under the confocal microscope at 20 dai ((a, d, and g) liquid inoculant, (b, e, and h) peat inoculant, and (c, f, and i) encapsulated inoculant). The magnification is the same in panels a, b, c, g, h, and I (bars = 200 µm), and in panels d, e, and f (bars = 50 µm). Significance at $p \leq 0.05$ is indicated by mean standard error bars (n = 3).

CHAPTER V

CONCLUSION

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 inoculant production.

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, which may be suitable for small scale of encapsulated inoculant production.

The shelf-life of most inoculants produced by pump model could maintained the cell number of inoculants at least for 6 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 L 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.

Three types of inoculant production by peristaltic- and syringe pumps model was increasing the soybean growth, plant biomass, and yield under field conditions. The plant with rhizobium inoculation showed effectively increased the total nitrogen content, N-fixed, and seed weight at 55, and 100 dai, respectively. The confirmation of strain USDA110 in the nodule was monitored by FA technique that showed green color under both fluorescent microscope and confocal microscope. Therefore, we can conclude that the inoculant production in this system could promote the soybean growth, plant biomass, and increasing soybean yield under the field condition. Therefore, this developed inoculant production system could be further used to produce high quality of bradyrhizobium inoculant with reduce the cost of production.

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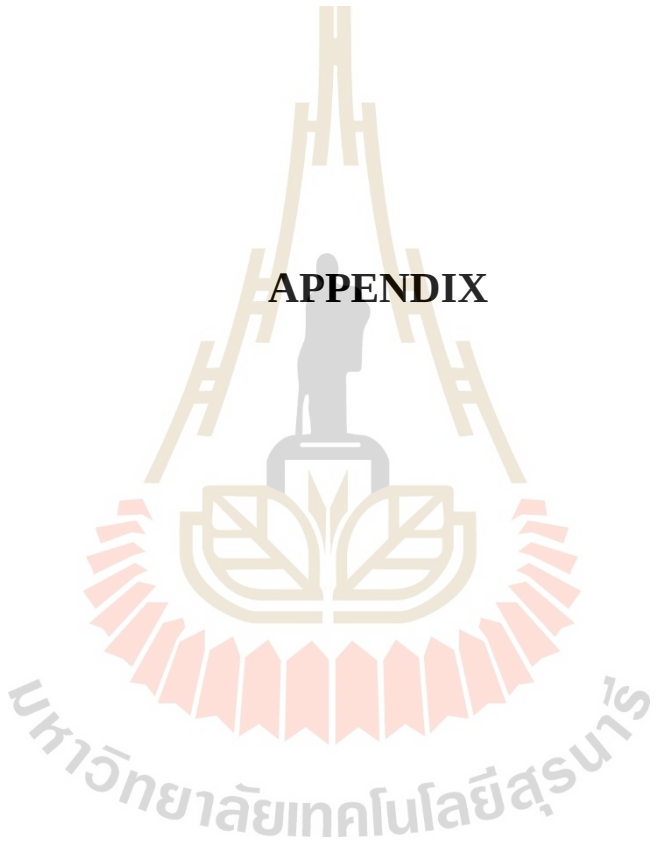
APPENDIX



Cost of inoculant production was composed of materials and chemical substances cost, package cost, depreciated cost of instruments, utilities cost, and labor cost.

Table A.1 Raw materials and chemical substances cost per 1 liter of liquid inoculant.

Raw materials and chemical substances	**Cost (Baht/kg)**	**Cost (Baht/g)**	**Numbers were used (g)**	**Cost (Baht)**
Mannitol	*6,395.48	6.40	10	63.95
KH_2PO_4	*4,120.96	4.12	0.5	2.06
NaCl	*3,700.00	3.70	0.1	0.37
$MgSO_47H_2O$	*1,793.38	1.79	0.2	0.36
PVP	*980.00	0.98	20	19.60
Total				**86.35**

*Price of chemical substances were referenced from catalogue of Fluka company (2020).

Table A.2 Raw materials and chemical substances cost per 1 liter of peat inoculant.

Raw materials and chemical substances	**Cost (Baht/kg)**	**Cost (Baht/g)**	**Numbers were used (g)**	**Cost (Baht)**
Peat	15.2	0.0152	650	9.88
YEM both	*66.74	0.0667	250	16.67
$CaCO_3$	10	0.0100	100	1
Total				**27.56**

*Price of chemical substances were referenced from catalogue of Fluka company (2020).

Table A.3 Raw materials and chemical substances cost per 1 liter of encapsulated inoculant.

Raw materials and chemical substances	Cost (Baht/kg)	Cost (Baht/g)	Numbers were used (g)	Cost (Baht)
Mannitol	*6,395.48	6.395	10	63.95
KH_2PO_4	*4,120.956	4.121	0.5	2.06
NaCl	*3,700	3.700	0.1	0.37
$MgSO_47H_2O$	*1,793.379	1.793	0.2	0.36
Sodium Alginate	*4,300	4.300	20	86.00
Skim milk	*980	0.980	7.5	7.35
Total				**160.09**

*Price of chemical substances were referenced from catalogue of Fluka company (2020).

Table A.4 Package for inoculant productions.

Inoculant types	Type of package	Number of package used in 1 L	Price of packaging/unit (Baht)	Total (Baht)
Liquid	Plastic bottle 125 ml	10	2.2	22
Peat	Plastic bag 7×11 inches	10	0.08	0.8
Encapsulated	Plastic bag 7×11 inches	10	0.08	0.8

Table A.5 Depreciated cost of instruments (based on 7 years) for liquid inoculant.

Instruments	Cost of instrument (Baht)	[a]Cost/hour (Baht)	Time used (min)	Cost (Baht)
pH meter	24,992	0.41	10	0.07
Shaker	240,000	3.91	7,200	469.2/[b]150 = 3.12
Autoclave	140,000	2.28	60	2.28/[b]10 = 0.228
Syringe pump	38,774.14	0.63	10	0.11/[b]10 = 0.011
Peristaltic pump	94,792.8	1.55	10	0.11/[b]10 = 0.011
Tube	3,500	0.06	10	0.01/[b]1 = 0.001
Laminar flow	600,000	9.78	10	1.63/[b]10 = 0.163
Total				**3.604**

[a]cost/hour = price of instrument/(7×365×24).

[b]number of materials can be used in one time of using.

Table A.6 Depreciated cost of instruments (based on 7 years) for peat inoculant.

Instruments	Cost of instrument (Baht)	[a]Cost/hour (Baht)	Time used (min)	Cost (Baht)
pH meter	24,992	0.41	10	0.07
Shaker	240,000	3.91	7,200	469.2/[b]150 = 3.12
Autoclave	140,000	2.28	60	2.28/[b]10 = 0.228
Syringe pump	38,774.14	0.63	10	0.11/[b]10 = 0.011
Peristaltic pump	94,792.8	1.55	10	0.11/[b]10 = 0.011
Tube	3,500	0.06	10	0.01/[b]1 = 0.001
Laminar flow	600,000	9.78	10	1.63/[b]10 = 0.163
Sealer machine	499	0.01	5	0.01/[b]10 = 0.001
Total				**3.605**

[a]cost/hour = price of instrument/(7×365×24).

[b]number of materials can be used in one time of using.

Table A.7 Depreciated cost of instruments (based on 7 years) for encapsulated inoculant.

Instruments	Cost of instrument (Baht)	[a]Cost/hour (Baht)	Time used (min)	Cost (Baht)
pH meter	24,992	0.41	10	0.07
Shaker	240,000	3.91	7,200	469.2/ [b]150 = 3.12
Autoclave	140,000	2.28	60	2.28/ [b]10 = 0.228
Syringe pump	38,774.14	0.63	10	0.11/ [b]10 = 0.011
Peristaltic pump	94,792.8	1.55	10	0.11/ [b]10 = 0.011
Tube	3,500	0.06	10	0.01/ [b]1 = 0.001
Laminar flow	600,000	9.78	60	9.785/ [b]10= 0.979
Sealer machine	499	0.01	5	0.01/ [b]10 = 0.001
Total				**4.525**

[a]cost/hour = price of instrument/(7×365×24).

[b]number of materials can be used in one time of using.

Utilities cost were composed of electricity cost and water

Table A.8 Electricity costs for liquid inoculant (1 unit = 4.4217 Baht).

Instruments	Power (kilowatt)	Time used (hour)	[a]Cost (Baht)
pH meter	0.005	0.17	0.004
Shaker	0.352	120	200.26/ [b]150 = 1.254
Autoclave	2	1	9.48/ [b]10 = 0.884
Syringe pump	0.48	0.17	0.38/ [b]10 = 0.036
Peristaltic pump	2.07	0.17	0.38/ [b]10 = 0.156
Laminar flow	0.093	0.17	0.09/ [b]10 = 0.007
Total			**2.332**

[a]Cost = Power (kw)×Time were used (h)×4.4217.

[b]number of materials can be used in one time of using.

Table A.9 Electricity costs for peat inoculant (1 unit = 4.4217 Baht).

Instruments	Power (kilowatt)	Time used (hour)	[a]Cost (Baht)
pH meter	0.005	0.17	0.004
Shaker	0.352	120	200.26/ [b]150 = 1.254
Autoclave	2	1	9.48/ [b]10 = 0.884
Syringe pump	0.48	0.17	0.38/ [b]10 = 0.036
Peristaltic pump	2.07	0.17	0.38/ [b]10 = 0.156
Laminar flow	0.093	0.17	0.09/ [b]10 = 0.007
Sealer machine	0.48	0.17	0.38/ [b]10 = 0.036
Total			**2.368**

[a]Cost = Power (kw)×Time were used (h)×4.4217.

[b]number of materials can be used in one time of using.

Table A.10 Electricity costs for encapsulated inoculant (1 unit = 4.4217 Baht).

Instruments	Power (kilowatt)	Time used (hour)	[a]Cost (Baht)
pH meter	0.005	0.17	0.004
Shaker (starter culture)	0.352	120	280.36/ [b]150 = 1.245
Autoclave	2	1	9.48/ [b]10 = 0.884
Syringe pump	0.48	1	2.27/ [b]10 = 0.212
Peristaltic pump	2.07	1	2.27/ [b]10 = 0.915
Sealer machine	0.48	0.17	0.38/ [b]10 = 0.036
Lamina flow	0.093	1	0.09/ [b]10 = 0.041
Shaker (inoculant)	0.352	168	200.26/ [b]30 = 26.148
Total			**29.486**

[a]Cost = Power (kw)×Time were used (h)×4.4217.

[b]number of materials can be used in one time of using.

Water cost = 10 Bahts/1,000 liters

Water used 2 liters were cost = (10×1)/1,000 = 0.01 Baht

Then utilities cost = 5.853 + 0.01 = 5.874 Bahts

Labor cost (Baht/h) = salary/(day in one month × working hour)

= 9,000/(30×8) = 37.5 Bahts/h

Therefore, the cost of production was calculated as indicate in table A.11.

Table A.11 Total cost of inoculant production.

List of costs were used for calculated	**Cost of production (Baht)/(kg or l)**		
	Liquid	**Peat**	**Encapsulated**
Raw materials and chemical Substances cost	86.35	27.56	160.090
Packaged cost	22	0.8	0.8
Depreciated cost of instruments	5.936	5.973	34.011
Utilitied cost	5.874	5.874	5.874
Labor cost	6.25	6.25	37.5
Total	**104.410**	**45.657**	**237.475**

BIOGRAPHY

Mr. Beedou Aphaiso was born on December 1st, 1991 in Borikhamxay, Lao PDR. In 2008, He graduated high school from Veingkham school, Borikhamxay. He graduated with the Bachelor of Agronomy, Faculty of Agriculture, National University of Laos. Then, he studied Master Degree at School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima, Thailand. He received a scholarship by the One Research One Graduate (OROG) of the Thailand Research Fund, December 23, 2017-October 22, 2019. During his Master Degree, he presented research work in the SUT International Virtual Conference on Science and Technology 2020 (IVCST 2020), August 28, 2020, Nakhon Ratchasima, Thailand (Presentation: Infusion starter culture-based as a model for pilot scale of bradyrhizobial inoculant production).