EFFECTS OF USING ENHANCED BIOFERTILIZER CONTAINING N-FIXER BACTERIA ON PATCHOULI GROWTH

NORHAZIMAH BINTI ABDUL HALIM

UNIVERSITI MALAYSIA PAHANG

UNIVERSITI MALAYSIA PAHANG

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EFFECTS OF USING ENHANCED BIOFERTILIZER CONTAINING N-FIXER BACTERIA ON PATCHOULI GROWTH

NORHAZIMAH BINTI ABDUL HALIM

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

April 2009

I declare that this thesis entitled “Effects of Using Enhanced Biofertilizer Containing N-fixer Bacteria on Patchouli Growth” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Special Dedication to my family

For all your care, support and believe in me.

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ABSTRACT

Producing effective biofertilizer is crucial for environment and future demands while patchouli is important essential oil plants in the world. The objective of this study was to evaluate the effect of enhancing biofertilizer with N-fixer bacteria by applying on patchouli plant. In this study, growth profile of screened N-fixer bacteria is obtained to harvest highest yield of active bacteria. Then, N-fixer strain is added in purchased biofertilizer using peat moss carrier. After 2 weeks, the nitrogen content is determined by Kjeldahl method. The effectiveness of this modified biofertilizer is indicated by average physical changes of plants. From analysis, it was found that nitrogen content of biofertilizer is increased linearly with the increase of N-fixer percentage inoculum. Using biofertilizer can offer yield as good as using chemical fertilizer if complex biofertilizer containing different strain is used. Application of enhanced biofertilizer on patchouli showed improvement of leaves and branch growth up to 8% and 5% respectively compared to original biofertilizer. The suitable range of N-fixer inoculum should be applied to biofertilizer is more than 10%.

ABSTRAK

Penghasilan baja bio yang berkesan adalah amat kritikal untuk alam sekitar dan permintaaan pada masa hadapan manakala patchouli diklasifikasikan sebagai tumbuhan berharuman penting di dunia. Objektif kajian ini adalah untuk menilai kesan penggunaan baja bio yang ditambahbaikkan dengan bakteria pengikat nitrogen dengan cara mengaplikasikannya terhadap pokok patchouli. Dalam kajian ini, profil pertumbuhan bakteria pengikat nitrogen ditentukan untuk memperolehi sebanyak mungkin bakteria yang aktif. Kemudian bakteria tersebut dicampur dengan pembawa lumut-gambut dan dibiarkan selama 2 minggu sebelum kandungan nitrogen dalam baja bio ditentukan dengan kaedah Kjeldahl. Keberkesanan baja bio ini dapat ditunjukkan dengan perubahan fizikal purata tumbuhan. Daripada analisis, didapati bahawa kandungan nitrogen di dalam baja bio meningkat dengan setiap peningkatan peratusan inokulum N-fixer. Penggunaan baja bio dapat memberi kesan sebaik baja kimia jika baja bio kompleks yang memgandungi pelbagai spesies digunakan. Aplikasi baja bio yang ditambahbaikkan terhadap patchouli menunjukkan 8% dan 5% peningkatan berlaku kepada pertumbuhan daun dan cabang berbanding baja bio yang asal. Had inokulum N-fixer yang sesuai diaplikasikan kepada baja bio adalah 10% dan keatas.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

DNS	-	Di-Nitro Salicylic Acid
DI	-	deionized
g	-	gram
g/L	-	gram per liter
K	-	potassium
L	-	liter
mg/L	-	milligram per liter
min	-	minutes
mL	-	mililiter
N	-	nitrogen
N	-	Normality
N-fixer	-	nitrogen fixer bacteria
nm	-	nanometer
OD	-	optical density
P	-	phosphorus
rpm	-	rotation or revolution per minute
v/v	-	volume per volume
v/w	-	volume per weight
w/v	-	weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

In this chapter we will discuss about background of study, objective, scope, problem statement and also rationale and significance of the research.

1.1 Background of Study

Fertilizer is widely used to supply essential nutrient for plant to increase yield. In fact, yields of most crop plants are increase linearly with the amount of fertilizer that they absorb (Loomis and Conner, 1992). Due to this fact, agricultural sector are strongly depending on fertilization with mineral nutrients. When crop plants are grown under modern high-production conditions, substantial amounts of nutrients are removed from the soil (Taiz and Zeiger, 2002). Then, to prevent deficiencies, nutrients can be added back to the soil in the form of fertilizers.

There are many types of fertilizer that existed such as inorganic fertilizer, organics fertilizer and biofertilizer. Fertilizers that provide nutrients in inorganic forms are called chemical fertilizers and those that derive from plant or animal residues are considered as organic fertilizers (Taiz and Zeiger, 2002). Biofertilizer are the products containing living cells of different types of microorganisms which have an ability to mobilize nutritionally important elements from non usable to usable form through biological process (NIIR Board, 2004). Among of these fertilizers, chemical fertilizer is the most extensively used in plantation.

Global fertilizer consumption is supported by an exponential growth in biofuels crops and a recovery in fertilizer use in the main consuming regions. Grain consumption is rising, driven by strong demand for food, feed and biofuels production, leading to very tight grain market conditions and a severe contraction of the world stock-to-use ratios well below critical levels (Maene, 2007).

However, extensive used of chemical fertilizers in long term cause declining in productivity and also environmental quality (Rahim, 2002). In the light of these problems, the use of organic fertilizers, biofertilizer and other microbial products is crucial in the current attempt to make the agriculture industry a viable component of a healthy and pleasant ecosystem (Rahim, 2002). Improvement should be done to these fertilizers to replace the usage of chemical fertilizer. Thus, this undergraduate research project is set up to improve the biofertilizer effectiveness to make it have superior potency over the chemical fertilizer.

1.2 Objective

The aim of this research is to modifiy and enhances biofertilizer by applying active nitrogen fixer (N-fixer) bacteria inside it. Thus, the objectives of this research are:

i. To obtain N-fixer growth profile in batch fermentation
ii. To obtain optimum N-fixer percentage inoculums can be applied in modified biofertilizer based on physical changes of plants.
iii. To compare the effect using enhanced biofertilizer containing different percentage of N-fixer inoculums with unmodified biofertilizer, chemical and organics fertilizer

1.3 Scope of Study

The scope of this research are to determine the effect of N-fixer inoculums in modified biofertilizer and compare it effectiveness with others fertilizer. Firstly, N-fixer growth profile is obtained by fermentation to gain active N-fixer. Then, N-fixer is added in biofertilizer with different percentage before applying on patchouli plants. Changes of nitrogen content in biofertilizer will be determined by Kjeldahl method. The effectiveness of enhanced biofertilizer will be tested on patchouli and proved by its physical changes. The techniques that will be employed include glucose assay, OD reading and physical changes observation. The equipment involved is UV-Vis Spectrophotometer, Kjeldahl apparatus and measuring set.

1.4 Problems Statement

The nature and the characteristics of nutrient release of chemical fertilizer, organic fertilizer and biofertilizers are different, and each type of fertilizer has its advantages and disadvantages with regard to crop growth and soil fertility (Chen, 2008). Chemical fertilizers are commonly used in plantation areas or farms because the nutrients are soluble and immediately available to the plants; therefore the effect is usually direct and fast (Chen, 2008). Besides, the price is lower and more competitive than organic fertilizer, which makes it more acceptable and often applied by users (Chen, 2008).

As a comparison, organic fertilizer production often has difficulties to standardize its constituent and the nutrient release rate is too slow to meet crop requirements in a short time, hence some nutrient deficiency may occur (Chen, 2008). Nevertheless, the usage of organics fertilizer does not leaved residues that can cause harm to environment like chemical fertilizer because they release nutrients slowly and contribute to the residual pool of organic N and P in the soil, reducing N leaching loss and P fixation (Chen, 2008). Chemical fertilizer does gives good yield but deteriorate our environment (Rahim, 2002).

The dilemma that fertilizer consumer face is to choose fertilizer that can give higher yield but cause harm to environment or to choose fertilizer that can preserve the environment but give slower effect. As a winding up, biofertilizer can be used to substitute organics and chemical fertilizer. Since it does not cause pollution like chemical fertilizer and give faster effect compare to organics fertilizer, it should be put at the first place. The technology for biofertilizers production is also relatively simple and installation cost is very low compared to chemical fertilizer plants.

However, the application of microbial fertilizers in practice, somehow, not achieved constant effect. The mechanism and interaction among the microbes still are not well understood, especially in real application (Wu *et al.*, 2004). The yields of plant that are using biofertilizer are still lower compare to using chemical fertilizer. Hence some modification needs to be done to enhance biofertilizer itself. This research is conducted to determine the effect of biofertilizer enhanced with N-fixer inoculums on patchouli growth. It is desire to obtain optimum percentage of inoculums to be added in biofertilizer within stated range.

1.5 Rationale and Significance

Fertilizers are global commodities and the price and availability are influenced by lot of factors throughout the world. In second half of 2008, it is predicted that South Asia, Eastern Europe and Central Asia will increase their fertilizer demand by more than 3% in 2008 and 2009 (Heffer and Prud'homme, 2008). Driven by strong demand for bio-fuels crops and a recovery of fertilizer use in the main consuming regions, global fertilizer consumption in 2006 rose 6 metric tonnes nutrient over 2005, to 161.8 metric tones (Maene, 2007).

At the regional level, the bulk of the increase in demand is expected to come from Asia and Latin America. South Asia and East Asia together would account for one third of total growth. In East Asia, regional demand will remain firm 2.9 % increase annually due to usage in China, Indonesia, Malaysia and Vietnam (Maene,

2007). As the major world palm oil producer, Malaysia and Indonesia is likely to have strong demand for fertilizer. Growth in the economies of China and India, in particular, as well as other countries has created a greater worldwide demand for fertilizers, and increased use of corn for ethanol production is increasing fertilizer demand in the United States due to expected increases in corn acres (Virginia State University, 2007).

World production capacity for nitrogen and phosphorus fertilizers is slightly greater than demand while potassium capacity is significantly greater than demand, but the production has been constrained by several factors in recent years including price (Virginia State University, 2007). As the base of the chemical fertilizers is fossil fuel, the prices of chemical fertilizer will goes up due to limited un-renewable resources. As a consequence, many countries are now shifting to use biofertilizer that have longer lasting effect compared to chemical fertilizer. Lot of researches and paperwork is done to fulfill world biofertilizer demand.

Some agreements that have been made by certain countries such as Kyoto Agreement in 1997 also give large opportunities for biofertilizer to be expanded. It is participated by 140 countries which agreed to reduce the usage of chemical fertilizer started by year 2010. Enhancement and large scale production of biofertilizer will helps to replace the usage of chemical fertilizer that can cause harm to environment and replace the usage of organics fertilizer that sluggish. This will definitely added the value of biofertilizer itself in the market and as a result, consumer will definitely choose biofertilizer instead of others fertilizer.

CHAPTER 2

LITERATURE REVIEW

This chapter will elaborate and summarize the literature reading for related major topics. Some explanation for the minor sub-topics is also included to give overall figure of this research.

2.1 Biofertilizer

2.1.1 History and Background

The term biofertilizer or called 'microbial inoculants' can be generally defined as a preparation containing live or latent cells of efficient strains of nitrogen fixing, phosphate solubilizing or cellulytic microorganisms used for application of seed, soil or composting areas with the objective of increasing the numbers of such microorganisms and accelerate certain microbial process to augment the extent of the availability of nutrients in a form which can assimilated by plant (NIIR Board, 2004). In large sense, the term may be used to include all organic resources (manure) for plant growth which are rendered in an available form for plant absorption through microorganisms or plant associations or interactions (NIIR Board, 2004).

The knowledge of applied microbial inoculums is long history which passes from generation to generation of farmers. It started with culture of small scale compost production that has evidently proved the ability of biofertilizer. This is recognize when the cultures accelerate the decomposition of organics residues and

agricultural by-products through various processes and gives healthy harvest of crops (Rahim, 2002). In Malaysia, industrial scale microbial inoculants are started in the late 1940's and peaking up in 1970's taking guide by *Bradyrhizobium* inoculation on legumes (Rahim, 2002). Government research institute, the Malaysian Rubber Board (MRB) had been conducting research on *Rhizobium* inoculums for leguminous cover crops in the inter rows of young rubber trees in the large plantations. Besides, Universiti Putra Malaysia (UPM) also has conducted many researches since 1980's on *Mycorrhiza* and initiated the research to evaluate the contribution of nitrogen from *Azospirillum* to oil palm seedlings (Rahim, 2002).

Rahim (2002) reported that *Mycorrhiza* inoculums are the biofertilizer that is increasingly being utilized and accepted in agriculture industry of Malaysia. It was also reported that a decomposer fungus *Trichoderma reesei*, combine with the nitrogen fixing bacterium *Azotobacter* produce compost within shorter time and give high nitrogen count. This can helps to fertilize unproductive sandy soil and spoiled land that result from mining activities because biofertilizer can helps to supply nutrient continuously. Large scale productions of biofertilizer are produced mainly for supplying nutrient, amelioration of toxic effect in soils, root pest and disease control, improved water usage and soil fertility (Rahim, 2002). Since the substrate for inoculate are abundant such as mine sands and agricultural wastes, the production cost is cheaper and environmentally safe.

There are lot of perception is lay on biofertilizer. It is often perceived to be more expensive than the chemical fertilizers due to the lack of skills and technology to produce biofertilizer products from abundant wastes (Rahim, 2002). Besides, the effect on the crops is slow, compared to chemical fertilizers. Special care such as storage or mixing with powders is also needed to handle microbial inocula to make they remain effective for extended use. As biofertilizers contain living organisms, their performance therefore depends on environment surrounding. Hence, outcomes are bound to be inconsistent (Rahim, 2002). Short shelf life, lack of suitable carrier materials, susceptibility to high temperature, problems in transportation and storage are biofertilizer bottlenecks that still need to be solved in order to obtain effective inoculation (Chen, 2008).

2.1.2 Production of Biofertilizer

There are several things need to be considered in biofertilizer making such as microbes' growth profile, types and optimum condition of organism, and formulation of inoculum. The formulation of inocula, method of application and storage of the product are all critical to the success of a biological product (Chen, 2008).

In general, there are 6 major steps in making biofertilizer. These includes choosing active organisms, isolation and selection of target microbes, selection of method and carrier material, selection of propagation method, prototype testing and large scale testing. First of all, active organisms must be decided. For example, we must decide to use whether organic acid bacteria or nitrogen fixer or the combination of some organisms. Then, isolation is made to separate target microbes from their habitation. Usually organism are isolate from plants root or by luring it using decoy such as putting cool rice underground of bamboo plants.

Next, the isolated organisms will be grown on Petri plate, shake flask and then glasshouse to select the best candidates. It is also important to decide form of our biofertilizer product wisely so that the right carrier material can be determined. If it is desired to produce biofertilizer in powder form, then tapioca flour or peat are the right carrier materials. Selection of propagation method is mainly to find out the optimum condition of organism. This can be achieved by obtaining growth profile at different parameter and conditions. After that, prototype (usually in different forms) is made and tested. Lastly, biofertilizer is testing on large scale at different environment to analyze its effectiveness and limitability at different surrounding.

2.1.2.1 Common Organism Used in Biofertilizer

Organisms that are commonly used as biofertilizers component are nitrogen fixers (N-fixer), potassium solubilizer (K-solubilizer) and phosphorus solubilizer (P-solubilizer), or with the combination of molds or fungi. Most of the bacteria included in biofertilizer have close relationship with plant roots (FNCA Biofertilizer Project Group, 2006). *Rhizobium* has symbiotic interaction with legume roots, and *Rhizo*-bacteria inhabit on root surface or in rhizosphere soil (FNCA Biofertilizer Project Group, 2006).

The phospho-microorganism mainly bacteria and fungi make insoluble phosphorus available to the plants (NIIR Board, 2004). Several soil bacteria and a few species of fungi posses the ability to bring insoluble phosphate in soil into soluble forms by secreting organic acids (NIIR Board, 2004). These acids lower the soil pH and bring about the dissolution of bound forms of phosphate.

While *Rhizobium,* Blue Green Algae (BGA) and *Azolla* are crop specific, bio-inoculants like *Azotobacter, Azospirillum,* Phosphorus Solubilizing Bacteria (PSB), Vesicular Arbuscular *Mycorrhiza* (VAM) could be regarded as broad spectrum biofertilizers (NIIR Board, 2004). VAM is fungi that are found associated with majority of agriculture crops and enhanced accumulation of plant nutrients (NIIR Board, 2004). It has also been suggested that VAM stimulate plant growth by physiological effects or by reducing the severity of diseases caused by the soil pathogens (NIIR Board, 2004).

Examples of free living nitrogen fixing bacteria are obligate anaerobes (*Clostridium pasteurianum*), obligate aerobes (*Azotobacter*), facultative anaerobes, photosynthetic bacteria (*Rhodobacter*), cyanobacteria and some methanogens. The example of K-solubilizer is *Bacillus mucilaginous* while for P-solubilizer are *Bacillus megaterium, Bacillus circulans, Bacillus subtilis* and *Pseudomonas straita* (Wu *et al.*, 2004).

2.1.3 N-Fixer Bacteria

Nitrogen fixer or N-fixers organism are used in biofertilizer as a living fertilizer composed of microbial inoculants or groups of microorganisms which are able to fix atmospheric nitrogen. They are grouped into free-living bacteria (*Azotobacter* and *Azospirillium*) and the blue green algae and symbionts such as *Rhizobium*, and *Frankia* and *Azolla* (NIIR Board, 2004).

Rhizobium inoculation is well known agronomic practice to ensure adequate nitrogen of legumes instead of N-fertilizer (NIIR Board, 2004). In root nodules the O_2 level is regulated by special hemoglobin called leghemoglobin. This globin protein is encoded by plant genes but the heme cofactor is made by the symbiotic bacteria. This is only produced when the plant is infected with *Rhizobium*. The plant root cells convert sugar to organic acids which they supply to the bacteroids. In exchange, the plant will receive amino-acids rather than free ammonia.

Azolla biofertilizer is used for rice cultivation in different countries such as Vietnam, China, Thailand and Philippines. Field trial indicated that rice yields are increased by 0.5-2 tonnes/hectare due to *Azolla* application (NIIR Board, 2004). *Azobacter* and *Azospirillum* can fix atmospheric nitrogen in cereal crops without any symbiosis while blue-green algae have been found to be very effective on the rice and banana plantation (NIIR Board, 2004).

2.1.3.1 Growth Condition of N-Fixer

The different strains of the same species may have different sensitivity on pH medium. N-fixer bacteria can develop on media with pH range from 5.5 -9.0. Each individual species are differing in their sensitivity to an acid medium. Moreover, it is said that the optimum pH of N-fixer is lies between ranges 7.2 to 8.2 (Mishustin and Shilnikova, 1969). At both acidic and alkaline pH range, the growth of the bacteria

will decrease (Dhanasekar, 2003). At this state, pH actually does affect the growth and nitrogen fixation.

Azotobacter is a typical mesophilic organism and most researchers regard 25-30ºC as the optimum temperature for *Azotobacter* (Mishustin and Shilnikova, 1969). At high temperature between 45-48ºC, vegetative *Azotobacter* cells cannot tolerate and will degenerated and dies (Mishustin and Shilnikova, 1969).

Growth condition can be experimented using shake flask. Generally, shake flasks experiment is used to optimize medium component while fermentor experiment is used to optimize operating condition.

2.1.4 Carriers Material for Biofertilizer

Biofertilizers are usually prepared as carrier-based inoculants containing effective microorganisms. Incorporation of microorganisms in carrier material enables easy-handling, long-term storage and high effectiveness of biofertilizers (FNCA Biofertilizer Project Group, 2006). Sterilization of carrier material is essential to keep high number of inoculant bacteria on carrier for long storage period. Gamma-irradiation or autoclaving can be used as method for sterilization.

Various types of material can be used as carrier for seed or soil inoculation. According to Somasegaran and Hoben (1994), the properties of a good carrier material for seed inoculation are inexpensive and available in adequate amounts. It must non-toxic to inoculants bacterial strain and non-toxic to plant itself. Because it acts as carrier for seed inoculation, it should have good moisture absorption capacity and good adhesion to seeds. Last but not the least; carrier should have good pH buffering capacity, easy to process and sterilized by either autoclaving or gamma-radiation. Table 2.1 summarizes the carrier materials that can be used for inoculation and its characteristics.

Table 2.1: Carrier materials for inoculants

Carrier Material	Inoculants Bacterium	Characteristics
Granular inoculant with nutrients	*Bradyrhizobium japonicum*	• Soil inoculation • Enhanced early nodulation
Alginate-perlite dry granule	*Rhizobium*	• Soil inoculation • Rhizobium strains survived in dry granules beyond 180 days. • The inoculant can be stored in a dry state without losing much viability.
Wheat bran, baggase of sugarcane	*Bradyrhizobium* and *Aspergillus niger*	• Soil inoculation • The number of cultured microorganisms was the highest with peat, followed by bran and sugarcane baggase.
Nutrient supplemented pumice	*Rhizobium*	• Seed inoculation • Good storage and handling properties and could be mixed directly with the seeds during the sowing process
Cheese whey grown cells in peat	*Rhizobium meliloti*	• Seed inoculation • Better survival at various temperature during storage, even under desiccation
Composted sawdust	*Bradyrhizobium* , *Rhizobium* and *Azospirillum*	• Seed inoculation • Good growth and survival of the inoculant strains.

Source: FNCA Biofertilizer Project Group (2006).

The most common way of inoculation is seed inoculation in which the inoculant (bacteria-carrier mixture) is mixed with water to make slurry-form, and then mixed with seeds (FNCA Biofertilizer Project Group, 2006). In this case, the carrier must be in a fine powder form. In order to achieve the tight coating of inoculant on seed surface, use of adhesive, such as gum arabic, methylethylcellulose, sucrose solutions, and vegetable oils, is recommended (FNCA Biofertilizer Project Group, 2006). Any locally available sticky material, which is non-toxic to bacteria and seeds, can be used as adhesive.

Seed inoculation may not always be successful due to low population or low survival of the inoculated bacterial strain on the seed surface and in the soil. In such instance, soil inoculation will be adopted, whereby a large population of a bacterial strain can be introduced into the soil. In soil inoculation, microbes are added directly to the soil where they have to compete with microbes already living in the soil that are already adapted to local conditions and greatly outnumber the inocula (Chen, 2008).

2.2 Microorganism Growth

Growth curves can be obtained by culturing microbe in batch modes. When a liquid medium is inoculated with seed culture, the organisms selectively take up dissolved nutrients from the medium and convert them into biomass (Shuler and Kargi, 2002). Growth will occurred and sample can be taken at certain interval to obtain growth curves. A typical growth curve in batch mode includes lag phase, logarithmic or exponential growth phase, deceleration phase, stationary phase and death phase. Figure 2.1 shows the normal bacteria growth curves that is adapted from Tortora *et al.* (2002).

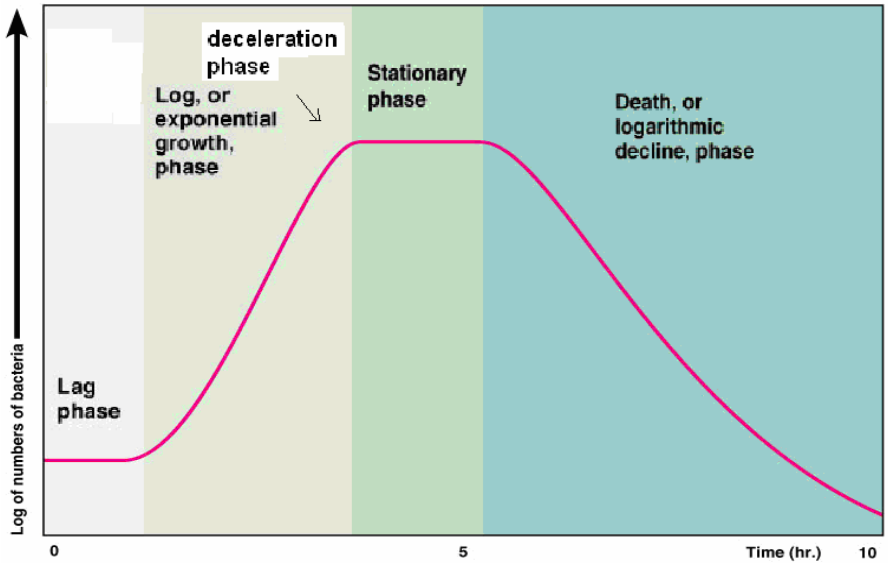

Figure 2.1: Bacteria growth curves

Source: Tortora *et al.* (2004)

Lag phase occurs immediately after inoculation and this is period of cells adaptation to a new environment where microorganism reorganizes their molecular constituent (Shuler and Kargi, 2002). Depending on the composition of nutrients, new enzymes are synthesized, the syntheses of some other enzymes are repressed, and the internal machinery of cells is adapted to the new environmental conditions (Shuler and Kargi, 2002). During this phase, cell mass may increase a little, without an increase in cell number density. Low concentration of some nutrient and growth factors may also cause a long lag phase (Shuler and Kargi, 2002).

The exponential growth phase is also known as logarithmic growth phase. In this phase, the cells have adjusted to their new environment. After the adaptation period, cell multiply rapidly, and cell mass and cell number density increase exponentially with time (Shuler and Kargi, 2002). This is period of balanced growth, in which all components of a cell grow at the same rate. During balanced growth, the net specific growth rate determined from either cell number or cell mass would be the same (Shuler and Kargi, 2002). Since the nutrient concentrations are large in this phase, the growth rate is independent of nutrient concentration (Shuler and Kargi, 2002). The exponential growth rate is first order.

The deceleration growth phase follow the exponential phase is when growths are decelerates due to either depletion of one or more essential nutrients or the accumulation toxic by-products of growth (Shuler and Kargi, 2002). The stationary phase starts at the end of the deceleration phase, when the net growth rate is zero or when the growth rate is equal to the death rate. Even though net growth rate is zero during the stationary phase, cells are still metabolically active and produce secondary metabolites (Shuler and Kargi, 2002).

The last phase is death phase which is also known as decline phase. Some cells death may start during stationary phase, and a clear demarcation between these two phases is not always possible since often dead cell lyses and intracellular nutrient released are used by living organism during stationary phase (Shuler and Kargi, 2002). If photometry method is used to determine cell density then, cell debris that is not removed from tank along the fermentation will deviate optical density reading thus upset accuracy for time determination of death phases.

2.3 Plant Nutrition

Mineral nutrients are elements acquired primarily in the form of inorganic ions from the soil. The large surface area of roots and their ability to absorb inorganic ions at low concentrations by active transport from the soil solution make mineral absorption by plants a very effective process.

There are only certain elements have been determined to be essential for plant growth. An essential element is defined as one that has a clear physiological role in plants (Epstein 1999). Plant nutrient can be divided into 2 major groups which is macronutrient and micronutrient. Table 2.2 shows the classification of plant mineral nutrients according to biochemical function and summarized the value of element level that may be required by plant.

Table 2.2: Classification of plant mineral nutrients according to biochemical function and level required.

Mineral	Functions	Concentration in dry matter (ppm)
Group 1	**Nutrients that are part of carbon compounds**	
Nitrogen	Constituent of amino acids, amides, proteins, nucleic acids, nucleotides, coenzymes, hexoamines, etc.	1.5
Sulfur	Component of cysteine, cystine, methionine, and proteins. Constituent of lipoic acid, coenzyme A, thiamine pyrophosphate, glutathione, biotin, adenosine-5′-phosphosulfate, and etc.	0.1
Group 2	**Nutrients that are important in energy storage or structural**	
Phosphorus	Component of sugar phosphates, nucleic acids, nucleotides, coenzymes, phospholipids, phytic acid, etc. Has a key role in reactions that involve ATP.	0.2
Boron	Complexes with mannitol, mannan, polymannuronic acid, and other constituents of cell walls. Involved in cell elongation and nucleic acid metabolism	20.0
Group 3	**Nutrients that remain in ionic form**	
Potassium	Required as a cofactor for more than 40 enzymes. Principal cation in establishing cell turgor and maintaining cell electroneutrality.	1.0
Chlorine	Required for the photosynthetic reactions involved in O_2 evolution.	100.0
Manganese	Required for activity of some dehydrogenases, decarboxylases, kinases, oxidases, and peroxidases. Involved with other cation-activated enzymes and photosynthetic O_2 evolution	50.0
Group 4	**Nutrients that are involved in redox reactions**	
Iron	Constituent of cytochromes and non-heme iron	2000.0

	proteins involved in photosynthesis,N_2 fixation, and respiration	
Zinc	Constituent of alcohol dehydrogenase, glutamic dehydrogenase, carbonic anhydrase, etc.	20.0
Copper	Component of ascorbic acid oxidase, tyrosinase, monoamine oxidase, uricase, cytochrome and etc.	6.0
Molybdenum	Constituent of nitrogenase, nitrate reductase, and xanthine dehydrogenase.	0.1

Source: Evans and Sorger (1966), Epstein (1972), Mengel and Kirkby (1987), and Epstein (1999).

2.3.1 Macronutrient

2.3.1.1 Nitrogen (N)

According to Taiz and Zeiger (2002), nitrogen is the mineral element that plants require in greatest amounts. It serves as a constituent of many plant cell components, including amino acids and nucleic acids.

Therefore, nitrogen deficiency rapidly inhibits plant growth. If such a deficiency persists, most species show chlorosis (yellowing of the leaves), especially in the older leaves near the base of the plant. Under severe nitrogen deficiency, these leaves become completely yellow (or tan) and fall off the plant. Thus a nitrogen-deficient plant may have light green upper leaves and yellow or tan lower leaves. Younger leaves may not initially show these symptoms because nitrogen can be mobilized from older leaves. When nitrogen deficiency develops slowly, plants may have markedly slender and often woody stems. This woodiness may be due to a buildup of excess carbohydrates that cannot be used in the synthesis of amino acids or other nitrogen compounds.

2.3.1.2 Phosphorus (P)

Phosphorus (as phosphate, $PO_4^{3-)}$ is an integral component of important compounds of plant cells, including the sugar–phosphate intermediates of respiration and photosynthesis, and the phospholipids that make up plant membranes (Taiz and Zeiger, 2002). It is also a component of nucleotides used in plant energy metabolism (such as ATP) and in DNA and RNA.

Characteristic symptoms of phosphorus deficiency include stunted growth in young plants and a dark green coloration of the leaves, which may be malformed and contain small spots of dead tissue called necrotic spots (Taiz and Zeiger, 2002). As in nitrogen deficiency, some species may produce excess anthocyanins, giving the leaves a slight purple coloration (Taiz and Zeiger, 2002). In contrast to nitrogen deficiency, the purple coloration of phosphorus deficiency is not associated with chlorosis (Taiz and Zeiger, 2002). In fact, the leaves may be a dark greenish purple. Additional symptoms of phosphorus deficiency include the production of slender stems and the death of older.

2.3.1.1 Potassium (K)

Potassium, present within plants as the cation K^+, plays an important role in regulation of the osmotic potential of plant cells. It also activates many enzymes involved in respiration and photosynthesis.

Taiz and Zeiger (2002) clarified that the first observable symptom of potassium deficiency is mottled or marginal chlorosis, which then develops into necrosis primarily at the leaf tips, at the margins, and between veins. Because potassium can be mobilized to the younger leaves, these symptoms appear initially on the more mature leaves toward the base of the plant. The leaves may also curl and crinkle. The stems of potassium-deficient plants may be slender and weak, with abnormally short internodal regions.

2.3.2 Micronutrient

Trace elements are the term used to describe nutrients required in very small quantities yet are still vital to plant growth. Below is the short description of trace element extracted from Taiz and Zeiger (2002).

2.3.2.1 Boron

Evidence suggests that it plays roles in cell elongation, nucleic acid synthesis, hormone responses, and membrane function. Boron deficient plants may exhibit a wide variety of symptoms, depending on the species and the age of the plant. A characteristic symptom is black necrosis of the young leaves and terminal buds. The necrosis of the young leaves occurs primarily at the base of the leaf blade. Stems may be unusually stiff and brittle. Structures such as the fruit, fleshy roots, and tubers may exhibit necrosis or abnormalities related to the breakdown of internal tissues.

2.3.2.2 Chlorine

The element chlorine is found in plants as the chloride ion (Cl^-). It is required for the water-splitting reaction of photosynthesis through which oxygen is produced. Plants deficient in chlorine develop wilting of the leaf tips followed by general leaf chlorosis and necrosis. The leaves may also exhibit reduced growth. Roots of chlorine-deficient plants may appear stunted and thickened near the root tips.

2.3.2.3 Manganese

Manganese ions (Mn^{2+}) activate several enzymes in plant cells. In particular, decarboxylases and dehydrogenases involved in the tri-carboxylic acid (Krebs) cycle are specifically activated by manganese. The best defined function of manganese is in the photosynthetic reaction through which oxygen is produced from water. The major symptom of manganese deficiency is intervenous chlorosis associated with the development of small necrotic spots. This chlorosis may occur on younger or older leaves, depending on plant species and growth rate.

2.3.2.4 Iron

Iron has an important role as a component of enzymes involved in the transfer of electrons (redox reactions), such as cytochromes. As in magnesium deficiency, a characteristic symptom of iron deficiency is intervenous chlorosis. In contrast to magnesium deficiency symptoms, these symptoms appear initially on the younger leaves because iron cannot be readily mobilized from older leaves. Under conditions of extreme or prolonged deficiency, the veins may also become chlorotic, causing the whole leaf to turn white. The leaves become chlorotic because iron is required for the synthesis of some of the chlorophyll–protein complexes in the chloroplast.

2.3.2.5 Zinc

Many enzymes require zinc ions ($Zn^{2+)}$ for their activity, and zinc may be required for chlorophyll biosynthesis in some plants. Zinc deficiency is characterized by a reduction in internodal growth, and as a result plants display a rosette habit of growth in which the leaves form a circular cluster radiating at or close to the ground. The leaves may also be small and distorted, with leaf margins having a puckered

appearance. These symptoms may result from loss of the capacity to produce sufficient amounts of the auxin indoleacetic acid.

2.3.2.6 Copper

Like iron, copper is associated with enzymes involved in redox reactions. The initial symptom of copper deficiency is the production of dark green leaves, which may contain necrotic spots. The necrotic spots appear first at the tips of the young leaves and then extend toward the leaf base along the margins. The leaves may also be twisted or malformed. Under extreme copper deficiency, leaves may abscise prematurely.

2.3.2.7 Molybdenum

Molybdenum ions (Mo^{4+} through $Mo^{6+)}$ are components of several enzymes, including nitrate reductase and nitrogenase. Nitrate reductase catalyzes the reduction of nitrate to nitrite during its assimilation by the plant cell; nitrogenase converts nitrogen gas to ammonia in nitrogen-fixing microorganisms.

The first indication of a molybdenum deficiency is general chlorosis between veins and necrosis of the older leaves. Flower formation may be prevented, or the flowers may abscise prematurely. Because molybdenum is involved with both nitrate assimilation and nitrogen fixation, a molybdenum deficiency may bring about a nitrogen deficiency if the nitrogen source is primarily nitrate or if the plant depends on symbiotic nitrogen fixation. Although plants require only small amounts of molybdenum, some soils supply inadequate levels. Small additions of molybdenum to such soils can greatly enhance crop or forage growth at negligible cost.

2.4 Total Nitrogen in Fertilizer

2.4.1 Methods Available

The determination of total nitrogen (N) in manure is extremely important if manure is to be used as a nutrient source for plants (Peters *et al.*, 2003). The determination of total N is a complicated process because of the presence of various forms of N (Kim, 2005). Total N is defined as the sum of organic-N and inorganic-N (NH_3-N, NH_4-N, NO_3-N and NO_2-N).

Several methods that are available to determine total N in soils are Kjeldahl method, Dumas method, Near Infrared Reflectance Spectroscopy (NIRS) and Direct Distillation method. However of the all methods used, the Kjeldahl method, which is wet oxidation method, is perhaps the most common methods for determination of total N (Kim, 2005). The dry Dumas method although older than the Kjeldahl method, has not been as widely adopted (Peters *et al.*, 2003). However, because of the development of computer automated instrument system that allow for more precise control of gases and improved instrument designed to handle more sample, the Dumas method has gained favor recently (Peters *et al.*, 2003).

These Dumas and Kjeldahl method have been used predominantly for the analysis of plant tissue and have been adapted for the determination of N in fertilizer (Peters *et al.*, 2003). Because there are various kind of fertilizer might available, an evaluation of method is important to ensure that determination is done correctly. Nitrogen analysis has remained difficult and expensive compared to the analysis of many other element, but with the advent of modern computers and microchip technology, both of these method are easier to use than in the past (Peters *et al.*, 2003). The selection of the method depends on many factors, of which cost, safety and ease of operation are vital.

2.4.2 Background of Kjeldahl Method

Nitrogen determination has a long history in the area of analytical chemistry. Johan Kjeldahl first introduced the Kjeldahl nitrogen method in 1883 at a meeting of the Danish Chemical Society (Labconco, 1998). As chairman of the chemistry department of the Carlsberg Laboratorium near Copenhagen, Kjeldahl was assigned to scientifically observe the processes involved in beer making. While studying proteins during malt production, he developed a method of determining nitrogen content that was faster and more accurate than any method available at the time. His method used simple equipment and could be performed by an inexperienced technician (Labconco, 1998).

Since 1883, the Kjeldahl method has gained wide acceptance and is now used for a variety of applications. Kjeldahl nitrogen determinations are performed on food and beverages, meat, feed, grain, waste water, soil and many other samples (Labconco, 1998). Hundred of papers have been written about the Kjeldahl procedure as numerous modifications have been proposed to speed the analysis and to improve precision, accuracy and N recovery (Jones, 2001). Depending on sample size, the Kjeldahl digestion procedure can be classified as either macro (1.0 gram or greater), or semi micro (1.0-0.5 gram), or micro (less than 0.5 gram), with the size of the digestion/ distillation apparatus scaled accordingly (Jones, 2001).

The major factor that influences the selection of a micro or semi-micro Kjeldahl method to determine organic nitrogen is its concentration. The macro-Kjeldahl method is applicable for samples containing either low or high concentration of nitrogen, but requires a relatively large sample volume for low concentrations. Semi-micro Kjeldahl method is generally applicable for sample that contain high concentration of nitrogen. Figure 2.2 and 2.3 are the figures of setting apparatus for macro-Kjeldahl and macro-Kjeldahl combination digestion/ distillation apparatus while Figure 2.4 shows setting apparatus for semi micro-Kjeldahl.

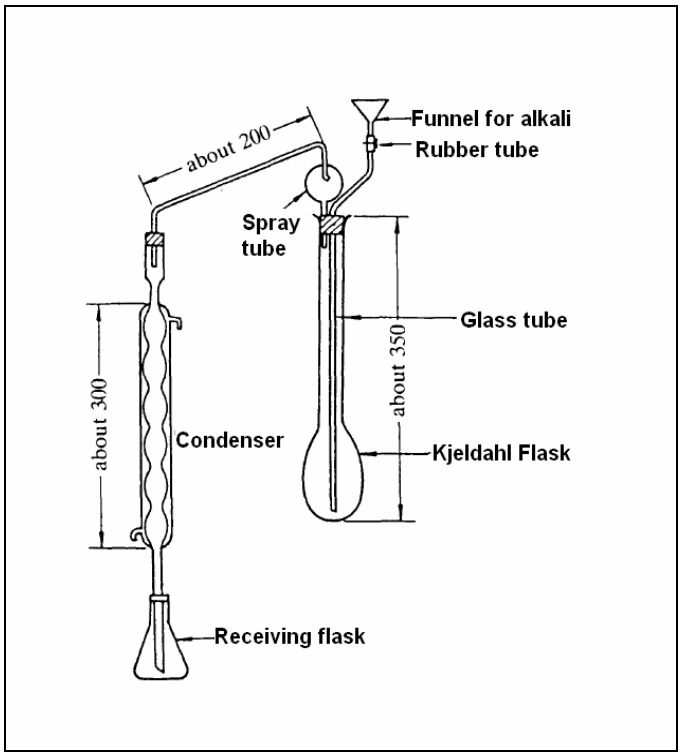

Figure 2.2: Setting apparatus for macro-Kjeldahl
Source: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf

Figure 2.3: Macro-Kjeldahl digestion and distillation apparatus
Source: Labconco (1998)

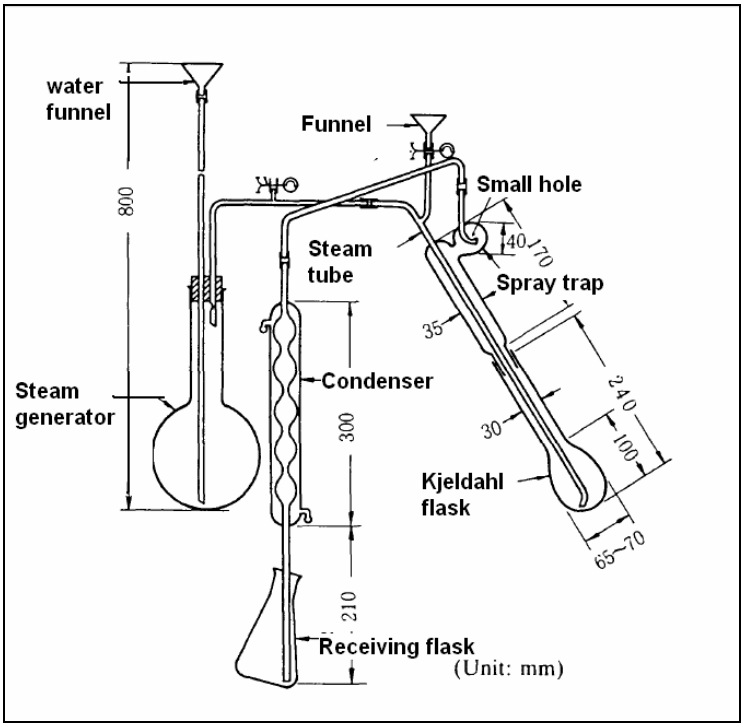

Figure 2.4: Setting apparatus for semimicro-Kjeldahl
Source: http://www.ffcr.or.jp/zaidan/FFCRHOME.nsf

Even though there are some disadvantages of Kjeldahl method such as requires concentrated acid and strong base, and need long digestion time and labor intensive procedure with a separate measurement for ammonium after digestion, it is still the best method. This is due to low cost of digestion/ distillation apparatus and can handle large sample sizes either wet or dry sample.

2.4.3 Principles of Kjeldahl Method

The main objective of the Kjeldahl method is to convert the nitrogen contained in materials to the ammonium form of nitrogen and then determine the concentration of ammonia-N (Peters *et al.*, 2003). In outline, the Kjeldahl method consist of three phase which are: 1) the digestion of the sample in sulfuric acid with a catalyst, which results in conversion of nitrogen to ammonia; 2) distillation of the

ammonia into a trapping solution; and 3) quantification of the ammonia by titration with a standard solution.

In the digestion phase, concentrated sulfuric acid is used to convert organically bound N to ammonium (NH^4). This is shown below.

$$\text{Organic N} + H_2SO_4 \longrightarrow (NH_4)2SO_4 + H_2O + CO_2 + \text{Sample by-products} \quad (1)$$

A digestion mixture is added to speed up the reaction (Dipak and Abhijit, 2005). The addition of the catalysts aids the chemical conversion while the addition of the salts elevates the temperature of the acid-sample mixture, speeding up the digestion (Peters *et al.*, 2003). Catalysts that have been used in the digestion process are mercury, copper, selenium, chromium and titanium (Simonne *et al.*, 1993). Mercury or mixture of selenium and copper are the most effective catalysts. However, because mercury and selenium is considered as environmental hazard, it is rarely used (Peters *et al.*, 2003). As a convenience to reduce bumping of the digestion mixture, alundum boiling chips or pumice are often added (Labconco, 1998). Adequate digestion time is important for complete conversion of organic N to NH_4 to occur. After the digestion mixture clears, and additional time period of two to three times the length of time it took for the mixture to clear is required to obtain complete N conversion (Jones, 2001). At clearing, about 92 to 93% of the organic N has been converted, and the additional boiling time is needed to obtain the remaining 7 to 8% (Jones, 2001).

In distillation phase, the NH_4^+ is converted to NH_3 by strong NaOH and distilled into trapping solution (usually using standard HCl solution). Reaction is shown below.

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3\,(\text{gaseous}) + Na_2SO_4 + 2H_2O \quad (2)$$

The majority of the NH_3 is distilled and trapped in the receiving acid solution within the first 5 or 10 minutes of boiling (Labconco, 1998). But depending on the

volume of the digestion mixture and the method being followed, 15 to 150 ml of condensate should be collected in the receiving flask to ensure complete recovery of nitrogen. Further extension of the distillation times and volumes collected simply results in more water being carried over to the receiving solution (Labconco, 1998). Excess water does not change the titration results.

Distillation times and distillate volumes collected should be standardized for all samples of a given methodology. The rate of distillation is affected by condenser cooling capacity and cooling water temperature, but primarily by heat input (Labconco, 1998). Typically the heating elements used for distillation have variable temperature controllers. A distillation rate of about 7.5 ml/minute is most commonly accepted (Labconco, 1998). Connecting bulbs or expansion chambers between the digestion flask and the condenser is an important consideration to prevent carryover of the alkaline digestion mixture into the receiving flask (Labconco, 1998). The slightest bit of contamination of the receiving solution can cause significant error in the titration step.

Last step which is quantification of ammonia is done by titrating trapping solution with standard solution. The ammonia is captured by a carefully measured excess of a standardized acid solution in the receiving flask. The excess of acid in the receiving solution keeps the pH low, and the indicator does not change. Reaction is shown below.

$$2NH_3 + 2H_2SO_4 \text{ (standard)} \longrightarrow (NH_4)_2SO_4 + H_2SO_4 \text{ (excess)} \quad (3)$$

The receiving solution should remain below 45°C during distillation to prevent loss of ammonia. The excess acid solution is exactly neutralized by a carefully measured standardized alkaline base solution such as sodium hydroxide. A color change indicated by suitable indicator such as mixture of methyl red and bromo-cresol green is produced at the end point of the titration.

$$(NH_4)_2SO_4 + H_2SO_4 \text{ (excess)} + 2NaOH \longrightarrow (Na)_2SO_4 + (NH_4)_2SO_4 + 2H_20 \quad (4)$$

2.5 *Pogostemon sp.*

2.5.1 Background

Patchouli or *Pogostemon sp.* is also known as Nilam in Malaysia and Indonesia. It is classified in Labiatae family and Angiosperm class. This plant was first described by botanists in the Philippines in 1845. Today growing interest in its fragrance has led to patchouli's widespread cultivation throughout tropical Asia. Indonesia is the major world exporter and contributes about 90 percent from total patchouli world oil (Nuryani *et al.*, 2005). Total export is 1.295 tonnes in 2002 with value about US $ 22.5 million (Nuryani *et al.*, 2005).

Patchouli is a small bushy herb which can grow up to one meter in height depending on species or variety. It is well branched, pubescent with quadrangular stem. Leaf shapes are ovate while petioles are 7 to 10 cm long. Patchouli that is grown in partially shaded areas will have wider, greener and thinner leaves but lower oil content. In vice versa, patchouli that is grown in open areas will have smaller, yellowish and thick leaves but higher oil content.

Even though there are more than 40 variety of patchouli but it is generally distinguish by its morphology, oil content and illnesses resistant to three common varieties (Nuryani et al., 2005). The common varieties are *Pogostemon cablin Benth,* (nilam Aceh), *Pogostemon heyneanus Benth* (nilam Jawa) and *Pogostemon hortensis Becker* (nilam sabun). Among of this three varieties, nilam Aceh or *Pogostemon cablin* is widely planted because it has high content and high quality of oil (Nuryani *et al.*, 2005). *Pogostemon cablin* might be introduced in Indonesia from Peninsular Malaysia or Philipina (Nuryani *et al.*, 2005). As it is propagated in Indonesia without control, probably changes on its original morphology or characteristic are occurred (Nuryani *et al.*, 2005).

Specific characteristic that differ *Pogostemon heyneanus* from *Pogostemon cablin* are leaves. *Pogostemon cablin* has smooth surface leaves while *Pogostemon*

heyneanus has rough surface leaves. *Pogostemon heyneanus* are more tolerant to nematode and pathogen due to high content of phenol and lignin. Figure 2.5 and Figure 2.6 are the figures of *Pogostemon cablin* and *Pogostemon heyneanus.*

Figure 2.5: *Pogostemon cablin*
Source: http://pics.davesgarden.com

Figure 2.6: *Pogostemon heyneanus*
Source: http://www.pantrygardenherbs.com

2.5.2 Environment Favor

Propagation of patchouli is usually done vegetatively (stem cuts). Patchouli should be provided with adequate shade during seedling stage because initially it will sag but recovers the following morning (RISE, 2000). Patchouli requires soil with

proper nutrition in order to obtain proper yields and better of quality. Soil should be supplemented with right dosage of fertilizers (depending on soil analysis) and the minerals element needed by the plants (RISE, 2000). The range temperatures are from 22 – 35^{o}C but the optimum temperature is 24 – 28^{o}C (Nuryani *et al.*, 2005). This plant thrives best in a damp and moist climate with an equally distributed rainfall of 2000 – 2500 mm/year (Nuryani *et al.*, 2005).

2.5.3 Properties

Patchouli oil is obtained by steam distillation of the leaves (Deguerry *et al.*, 2006). At present, the only commercial source of patchoulol synthetic routes for enantiomeric pure patchoulol is from patchouli plants (Deguerry *et al.*, 2006). Table 2.3 shows the physical and chemical properties of patchouli alcohol while Figure 2.7 shows the molecular structure of patchouli alcohol.

Table 2.3: Properties of patchouli alcohol

Type of Properties	Properties	Value
Physical	Vapor Pressure	<0.001 mm Hg (20^{o}C)
	Water Solubility	42.87 mg/L (25^{o}C)
Chemical	Molecular Weight	222.87
	Formula	$C_{15}H_{26}O$

Source: Bhatia *et al.* (2008)

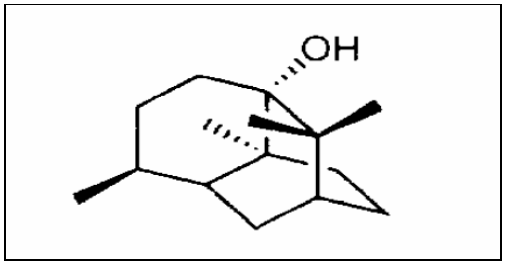

Figure 2.7: Molecular structure of patchouli alcohol

2.5.4 Usage

This essential oil is widely appreciated for its characteristic pleasant and long lasting woody, earthy, camphoraceous odor (Deguerry *et al*., 2006). Steam distillation of dried patchouli leaves yield as essential oil commonly called as 'Oils of Patchouli'. This oil is considered as one of the most important oils of the perfume industries (RISE, 2000). It blends well with other essential oils like vetiver, sandalwood, paranium and lavender. Because of its unique quality, it is used as fixative in a large number of high grade perfumes (RISE, 2000).

Historical records reveal that more than hundred years ago Indian export fabrics used to be heavily perfumed with patchouli leaves, a technique later adopted by the French manufacturers to create typical oriental aroma for their home spun shawls (Singh and Rao, 2008). Fresh patchouli leaves when crushes along with gugo and lemon grass serve as shampoo of old folks and even modern ladies (RISE, 2000). Patchouli alcohol is also ingredient used in cosmetics, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents (Bhatia *et al*., 2008).

Dry patchouli leaves have also been found to possess moth repellent properties and therefore, are used to scent wardrobes and protecting clothes especially woolens from insect damage (Singh and Rao, 2008). Crushed leaves and tops are used to repel cockroaches, moths and even leeches (RISE, 2000).

Patchouli oil is known to possess antifungal properties and is being used for skin infections, dandruff and eczema. In aromatherapy patchouli oil is recognized for its antidepressant, anti-inflammatory, cytophylactic, deodorant and fungidical properties (Singh and Rao, 2008).

CHAPTER 3

METHODOLOGY

This chapter will listed all method use starting from transferring N-fixer from slant bottles (mother culture) until applying on plants. The method of analysis to fulfill scope and objectives is also discussed. Basically, experiment is divided to two (2) major parts. In the first part, fermentation is done in shake flask to determine N-fixer fermentation profile. For the second part, purchased biofertilizer will be modified using N-fixer that is harvested from shake flask. All the fertilizer will be tested on patchouli plants.

3.1 Medium Preparation

Two types of medium are used throughout this research which is nutrient agar (NA) and nutrient broth (NB). Nutrient agar plate is prepared by mixing the nutrient ingredients in proportion to the amount required in flask. Flask is then covered with aluminum foil and autoclaved for 15 min. The plates are leaved undisturbed until the agar solidifies. About 10 g of glucose is added in NB to optimize growth in fermentation based on *Wu et al.* (2004) while unmodified NB is prepared for the other part. All NB is kept in refrigerator at 4^{o}C after it is autoclaved at 121^{o}C (20 minutes) for later use.

3.2 Maintenance of N-fixer

Culture of N-fixer is maintained along this research on agar plate and agar slant. Replication is done and N-fixer is kept dormant in refrigerator at 4^{o}C. Any contamination such as undesired strain is detected by streaking liquid suspension or cultures on new sterile agar plate. Colonies that grow on the agar is observed using naked eyes and microscope to detect contamination (existent of undesired bacteria colonies).

3.3 N-Fixer Fermentation Profile

Figure 3.1 show the flow work to determine the N-fixer fermentation profile.

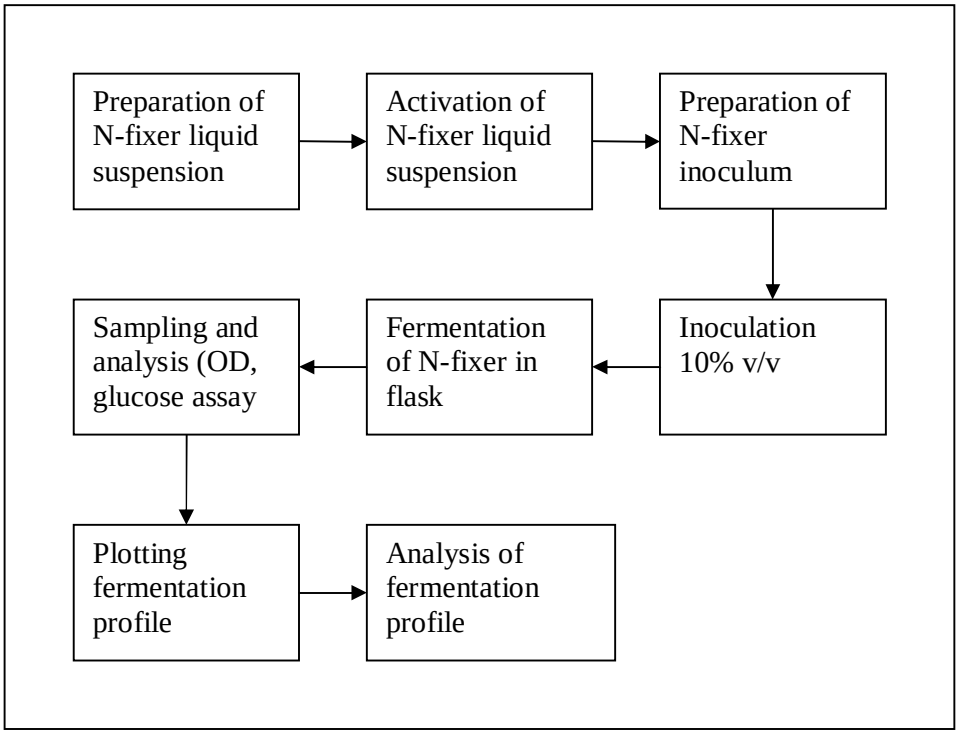

Figure 3.1: Flow process for determination of N-fixer profile.

Firstly, N-fixer from stock culture is streaked on new agar plate to obtain pure culture. After 3 days incubation at 33^{o}C, 2-3 loop of culture is transferred from

agar plate into 100 ml of sterile NB in shake flask. All procedures are done aseptically and experiment is run duplicate for all parts. Initial pH is fixed at pH 7.4 while shake flask will be shake at 200 rpm and 29^{o}C along the experiment.

3.3.1 Activation and Inoculation

Culture activation is done by transferring 10 % (v/v) of liquid suspension into fresh sterilized NB in shake flask. The mixture will be incubated in incubator shaker at 200 rpm and 29^{o}C for 30 hours.

Inoculation step is done by transferring 10 % (v/v) of liquid suspension from activation part into fresh sterilized NB in shake flask. It is then incubated for 18 hours at 200 rpm and 29^{o}C in incubator shaker.

3.3.2 Fermentation

Fermentation is started by transferring 10% (v/v) of inoculum suspension into sterilized modified NB. Sampling is done at certain interval for total 60 hours. Two-mL of sample is taken every 3 hour for the first 30 hours while for the last 30 hours, sample is taken every 6-9 hours depending on ease. Glucose content is determined from each sample using glucose assay while N-fixer growth is represent by optical density using spectrometry analysis (UV-VIS spectrophotometer). The growth conditions used along total 60 hours fermentation are 200 rpm and 29^{o}C. Initial pH is fixed at 7.4.

3.4 Enhancement of Biofertilizer

Fermentation process will be stopped at exponential phase to obtain highest yield of active cell for biofertilizer enhancement procedure. Figure 3.2 show the flow process to enhance biofertilizer.

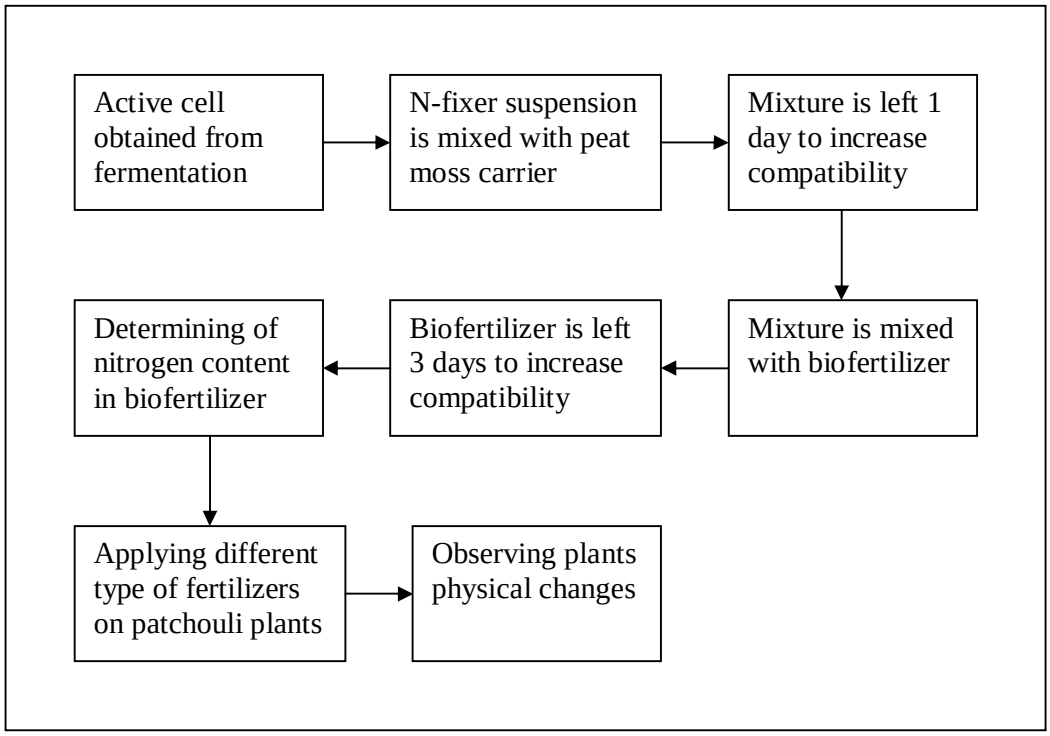

Figure 3.2: Flow process for producing enhanced biofertilizer.

3.4.1 Preparation of Enhanced Biofertilizer

Peat moss was used as the carrier for soil inoculation. First it was oven-dried at 60^{o}C for 72 hours, and then ground and sieved before it is autoclaved at 121^{o}C for 40 minutes. Ratio 0.125 g/g of peat moss is used for biofertilizer enhancement with N-fixer.

Active N-fixer bacteria from fermentation are transferred to sterilized peat moss, mixed until homogenized and then left for one day for adaptation. Various percentages of volumes per weight (v/w) of N-fixer are used as parameter which is 5%, 10%, 15% and 20 % v/w. Mixture of biofertilizer and N-fixer suspension in peat moss is left for 3 days to allow interaction between different types of organisms.

Testing on presence of N-fixer is done to identify that N-fixer is survived after the interaction. Sterile loops are stabbed into samples (modified biofertilizer) and streaked on sterile agar plate. The agar plate contain suitable medium for N-fixer growth but inhibit growth of *Mycorrhiza* and *Trichoderma reesei.* Control agar plate for *Mycorrhiza* and *Trichoderma reesei* is made by streaking sterilized peat moss on agar plate to ensure that no others microorganism are presence. The control agar plate (unstreaked sterile agar) is incubated together with all streaked agar plate for 2 days at 34^{o}C.

3.4.2 Poly Bag Experiment

Four replicate sets (A, B, C and D) of patchouli plants were used to observe the effectiveness of modified biofertilizer by observation on its physical changes. Each set of patchouli plants contain eight poly bags that will undergoes different treatment/ parameter. Total poly bag used along this experiment are 32 pots. A set of chilies plant was prepared to act as backup experiment.

Each of the plant in the sets is treated with chemical fertilizer, organics fertilizer, unmodified biofertilizer and modified biofertilizers with 5%, 10%, 15% and 20% of N-fixer suspension. There is no treatment for control poly bag. Amount of fertilizer put down are 100 g each for organic fertilizer and biofertilizer (modified and unmodified). For chemical fertilizer, only 10 granules are added. All fertilizer is applied at 2 weeks interval. Poly bags are watered at night every 2 days with pipe water. Data needed are recorded at 2 weeks interval for 8 to 10 weeks of experiment as in Table 3.1.

Table 3.1: Observation for physical changes of plant

Parameter	Measurement (cm)											
	Date A			Date B						Total Δ		
	Initial			Reading			Changes(Δ)					
Control												
Chemical												
Organic												
Biofertilizer 0%												
Biofertilizer 5%												
Biofertilizer 10%												
Biofertilizer 15%												
Biofertilizer 20%												

Figure 3.3 show the summary of overall experimental work for this project.

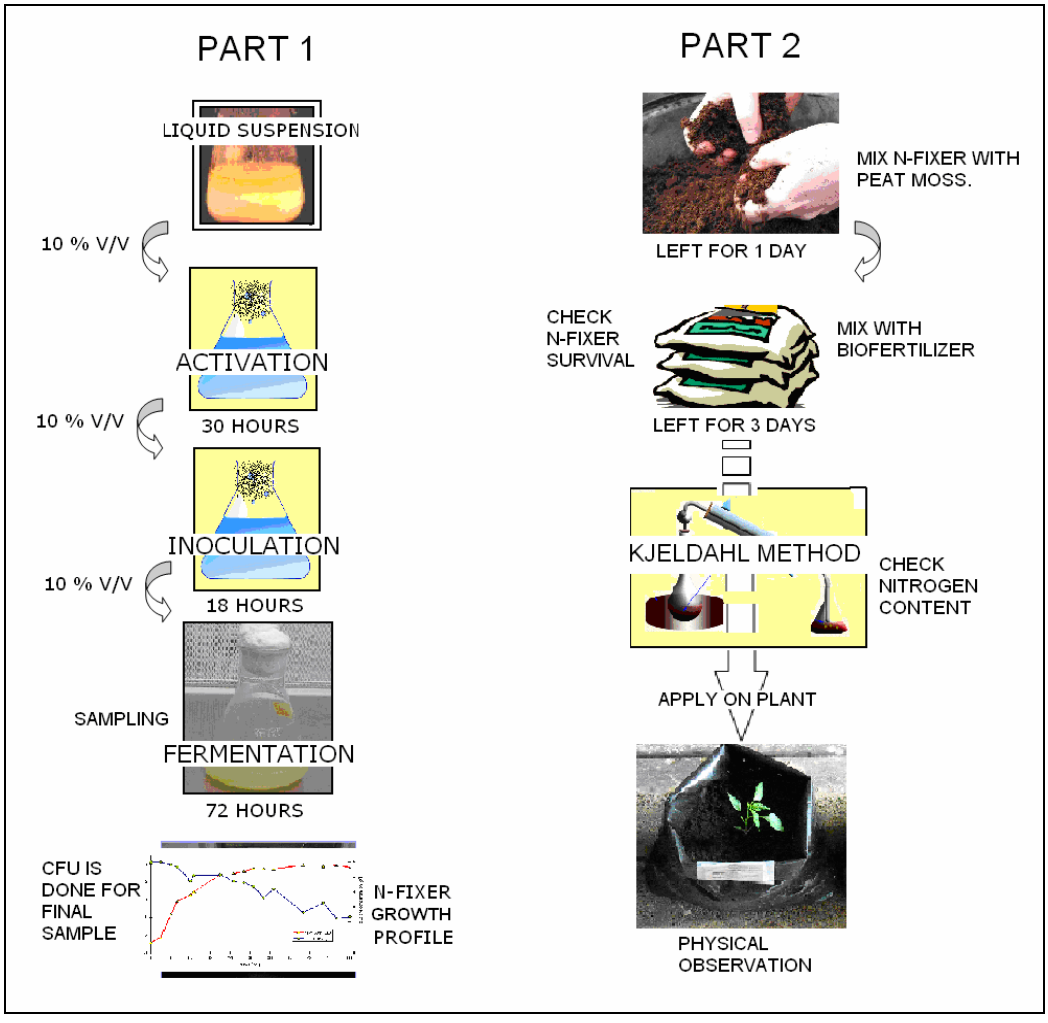

Figure 3.3: Summary of overall study

3.5 Analysis Method

3.5.1 Glucose Assay

Glucose assay by Miller (1959) is used to determine glucose concentration during fermentation. Same mixture ratio 1:2:10 (sample: DNS reagent: distilled water) is used for sample and glucose standard. 0.2 mL of DNS reagent is added to 0.1 mL of sample in test tube before it is capped with parafilm.

Next, test tube is boiled in 90^{o}C water for 5 minutes to develop red-brown color. After it is cooled, 1 mL of distilled water is added and mixture is shaken vigorously. Finally, OD is read at 550nm. If value is exceeding 2.00, dilution is done by mixing 1 part of that final sample with 9 part of distilled water until reading are below 2.00.

For standard glucose, stock solution is prepared by well mixed 1 gram of pure dextrose glucose with 1000 mL of distilled water. Stock solution is then mixed with some proportion of distilled water such as in Table 3.2 below.

Table 3.2: Proportion for glucose standard

Test Tube	Volume (mL)							
	1	2	3	4	5	6	7	8
Stock Solution	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Distilled Water	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3
DNS Reagent	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

The same procedure with glucose sample is repeated except that 10 mL of distilled water is added to test tube instead of 1 mL. Data for OD reading and glucose consumption versus time is plotted as growth profile.

3.5.2 Colony Forming Unit

After stopping fermentation at decided time, colony forming unit (CFU) is done to determine initial concentration of N-fixer that will be further used in making modified biofertilizer. Technique that will be applied are spread plate technique where a small amount of a previously diluted specimen is spread over the surface of a solid medium using a spreading rod.

The solution needed is 0.8% w/v saline to make specimen static in place. All the agar plate is labeled with the dilution number used. Initial sample of N-fixer after fermentation is diluted by transferring 10% v/v of sample to sterile saline in first capped tubes to produce sample with dilution 10^{-1}. Then, 10% v/v of solution from first capped tubes is transferred into second capped tubes containing sterile saline. This procedure is repeated until 20^{th} of dilution using different pipette tips for each tube. All tubes are swirled to mix well. Next, 0.01 mL of suspension is transferred to the middle of the agar and spread properly on the surface using the sterile glass spreader. Sample is replicated for all dilution numbers. All agar plates are incubated for a day. Only plates that contain 30 – 800 of colony present on agar are counted. CFU is calculated using equation below.

$$\text{CFU} = \text{Colonies/ mL} = \frac{(\text{no. of colonies observed})}{(\text{Volume used, mL})\ (\text{dilution})}$$

3.5.3 Nitrogen determination by Kjeldahl Method

Nitrogen content in biofertilizer before and after addition of N-fixer is determined using Kjeldahl method. Figure 3.4 shows the flow process for Kjeldahl method adapted from Jones (2001).

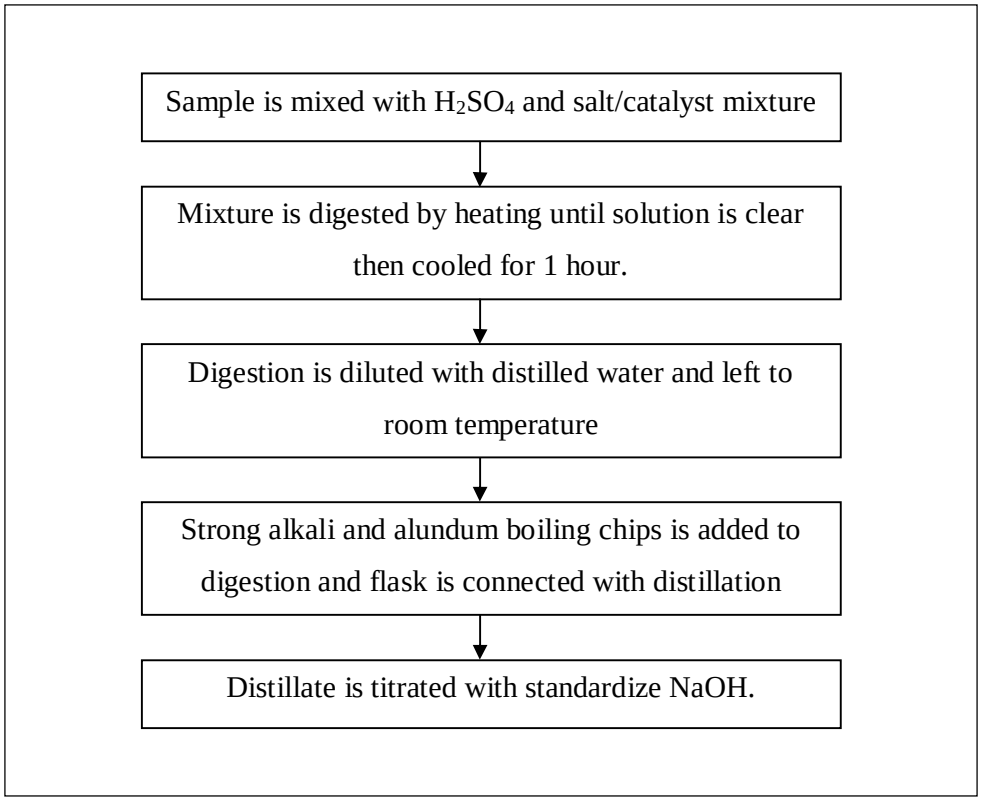

Figure 3.4: Flow process for Kjeldahl method

3.5.3.1 Preparation for Kjeldahl Method

Macro-Kjeldahl method is used to determine nitrogen content in biofertilizer. Material and reagent needed are 0.3 **N** sulphuric acid (H_2SO_4), 95% H_2SO_4, 0.1 **N** sodium hydroxide (NaOH), 40% NaOH, digestion salt mixture and indicator.

Concentrated 95% H_2SO_4 is directly taken from bottle while 0.3 **N** H_2SO_4 is prepared by diluting 8.4 mL of concentrated 95 % H_2SO_4 in 1 L of DI water. NaOH with 40% concentration is prepared by dissolving 400 g of tablet NaOH in 1 L DI water. For 0.1 **N** NaOH, 4 g of tablet is dissolved in 2 L of DI water.

Digestion salt/catalyst mixture is prepared by mixing 250 g of potassium sulphate with 8 g copper sulphate anhydrous. It is then well mixed and packed into single 15 g per packets.

3.5.3.2 Procedure of Kjeldahl Method

Homogeneity is increased by milling coarse samples. Accurately 5 g of sample are transferred into 500 mL of Kjeldahl flask. Next, 15 g of salt/catalyst mixture and 20 mL concentrated H_2SO_4 is added. Flask is then put on heating mantle and rotated frequently. Before that, heating input must be standardize and test its capability to boil 250 mL of 25^{o}C water in 5 minutes.

After solution is clear (light green-blue solution), digestion is continue for 1 hour to recover the remaining nitrogen. Solution is left to cool for 1 hour. Next, 250 mL of distilled water is added and again solution is left to cool to room temperature.

Erlenmeyer receiving flask is prepared by adding 10 mL of 0.3 **N** standardized H_2SO_4, 10 drops of indicator and 100 mL of distilled water. It is placed under condenser and water flow.

Subsequently, 2 to 3 alundum boiling chips are added into 70 mL of NaOH 40% in digestion flask. It is important to dispense NaOH using long funnel to avoid initial mixing that can cause NaOH accidentally entering receiving flask. Heating is continued until 100 to 150 mL of distillate is collected. All the distillates are titrated with 0.1 **N** NaOH to end-point. Volume need for titration is recorded. Procedures are repeated for blank except there are no sample is added. Figure 3.5 and 3.6 shows complete modified set of Kjeldahl apparatus and color development after titration.

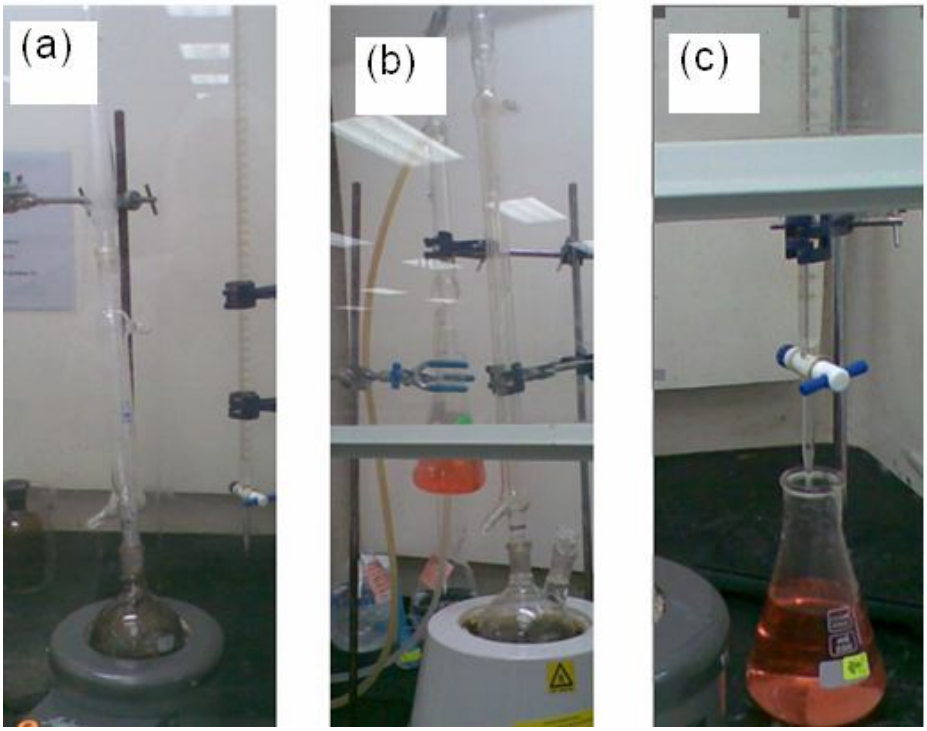

Figure 3.5: Modified set of Kjeldahl apparatus, a) digestion process and b) distillation process and c) titration process.

Figure 3.6: Color development after titration.

Total nitrogen content is determined by calculation below.

% of N (Total Kjeldahl Nitrogen)

$$= [(B_{mL} - A_{mL})/C_g] \times Nmeq/mL \times Dmg \times g/1000mg \times 100$$

Where A = Volume of NaOH sample titration (mL)

B = Volume of NaOH blank titration (mL)

C = Sample weight (5 g)

D = Miliequivalent weight of nitrogen (14 mg)

N = Normality of NaOH (0.1 **N**)

In winding up,

$$\% N = (B_{mL} - A_{mL}) \times 0.028$$

3.5.4 Physical Observation

The observation on total number of leaves, total branches and height were made for 8 to 10 weeks of experiment duration at 2 weeks interval. Total number of leaves is defined as the total number of leaves view during observation minus initial number of leaves. Only leaf that have diameter more than 1 cm is counted. The branch is counted if its length from original stem is more than 1 cm. Lastly, height is measured from soil surface to growing tip of the plant. All the data were recorded and plotted in suitable graph and analyzed into a conclusion.

CHAPTER 4

RESULTS AND DISCUSSION

This chapter will be discussed the result of all experimental work for this project.

4.1 N-Fixer Growth Profile

Analysis of growth profile is essential to determine the time when highest yield of active bacteria can be harvested. Knowing the right time to harvest bacteria is significant to reduce cost and produce active biofertilizer. Data that was recorded along 60 hours of fermentation is plotted in graph shown by Figure 4.1.

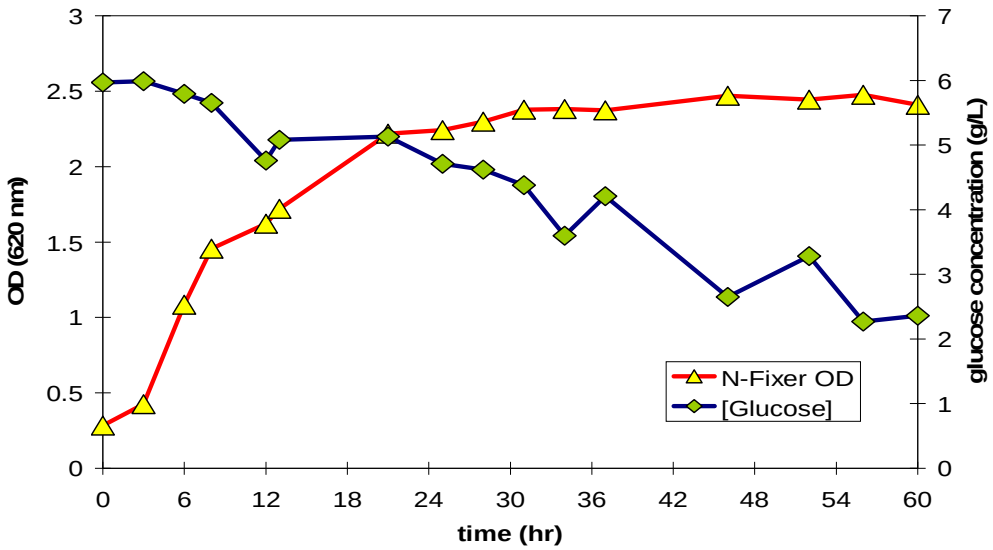

Figure 4.1: Growth profile for 60 hours fermentation

From growth profile, it is assumed those lag phases are occurred within less than 1 hour after bacteria were transferred into medium before exponential phase precede. This due to active cells and large inoculum size (10% v/v) used, and adaptation during activation and inoculation part. Shuler and Kargi (2002) have stated that duration of lag phase can be minimize by adapting cells to growth medium and conditions before inoculation, and using large inoculum size (5-10%).

Exponential phase is started after lag phase until 20 hours of fermentation. During this phase, bacteria cell multiply rapidly. Since there is no direct method to distinguish active bacteria, based on Shuler and Kargi (2002), it is assumed that bacteria are mostly young and active during exponential phase. Stationary phase is started at 35 hours fermentation followed by death phase which is begun at 46 hours. Once reading is dropped, it is assumed as starting of cell death even though clear demarcation between these two phases is not always possible.

Glucose concentration dropped as fermentation proceeds. This proved that glucose is being used by N-fixer for its growth. At the end of 60 hours, glucose concentration that is left is approximately 2 g/L. Initially, 10 gram of glucose was added in NB followed previous study by Wu *et al.* (2004). Conversely, after using glucose assay method it is discovered that initial glucose concentration reading is only approximately 6 g/L. This might due to mechanical error cause by incalibrated micropipette and UV-Vis spectrophotometer. The obtained glucose standard also give lower reading compared with previously done by Bashori (2008). There were probabilities that the glucose used was impure, mistake when making DNS reagent or dilution occurred during autoclaving.

Figure 4.2 shows the concentration of glucose before autoclaving, after autoclaving and after centrifugation. From graph, it is clearly shown that concentration changes are not significant and thus, it is concluded that no dilution were occurred during autoclaving and centrifugations.

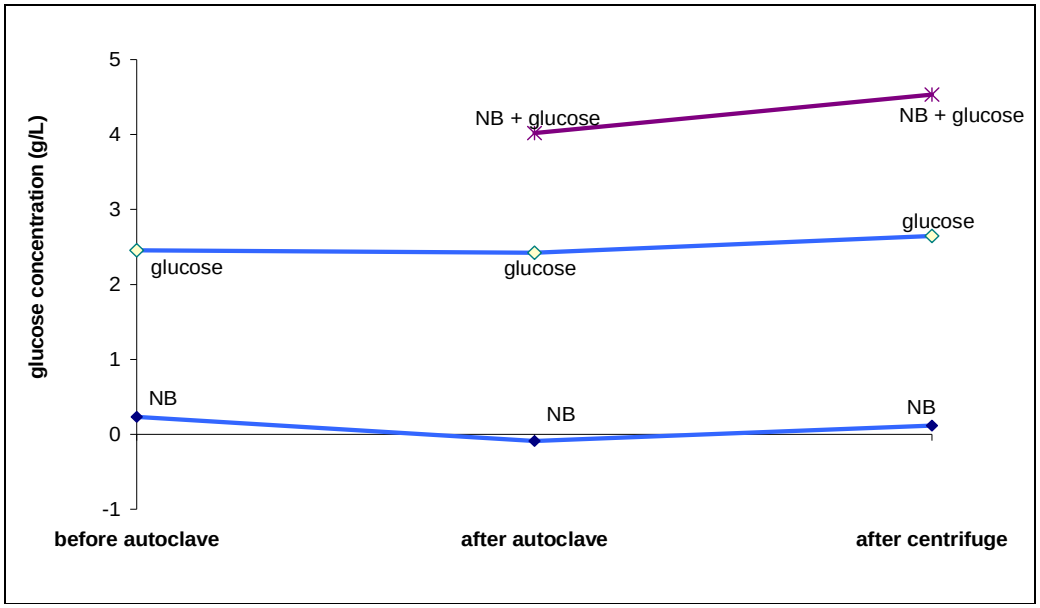

Figure 4.2: Concentration of glucose in medium. NB and glucose refers to nutrient broth and glucose solution (5 g/L) that undergoes autoclaving and centrifugation separately while NB + glucose refer to mixture of nutrient broth and glucose solution that undergoes autoclaving separately and centrifugation together.

Contamination test were made to prove that only N-fixer is existed in harvested bacterial suspension for biofertilizer making. It was done by streaking suspension on sterile agar plate to obtained pure culture. As the result, zero growth of unwanted strain was observed and this proved that no contamination was occurred. Figure 4.3 shows the result of plate streaking where only pure culture of N-fixer is present.

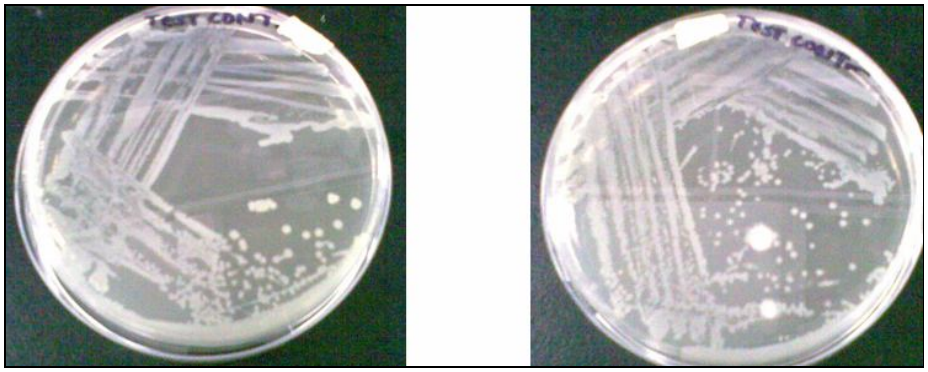

Figure 4.3: Testing on contaminant for replicate 1 and 2

4.2 Enhancement of Biofertilizer

4.2.1 Colony Forming Unit (CFU)

The range for count of cell to be used in enhancement of biofertilizer is 1- 20 x 10^{20}colonies/mL. The data of CFU was summarized in Table 4.1.

Table 4.1: Data for CFU

No. of colonies	Volume used (mL)	Dilution	CFU (colonies/mL)
208	0.01	10^{-16}	2.08 x 10^{20}

4.2.2 N-Fixer Test

Presences of N-fixer in biofertilizer were tested after 2 week to ensure that microbes are still active. This is important since there is no investigation on the relationship of N-fixer with existing microorganism in original biofertilizer. It is expected that competition might occurred between existed microorganisms with added N-fixer which will reduce the effectiveness of biofertilizer.

From observation, it was clearly proved that there were no microorganism growth on peat moss plate and biofertilizer 0% plate. This is fit with the fact that there are no N-fixer is added in peat moss (initial) and biofertilizer 0%. Analysis on morphology of pure culture on the remaining plate can be concluded that only N-fixer is grow. Since *Mycorrhiza* and *Trichoderma* already present in original biofertilizer (biofertilizer 0%) without modification except for addition of 150 g of peat moss to give similar structure and benefit with others, it is concluded that both fungi were unable to grow on plain medium. From this experiment, it is concluded that N-fixer are capable to maintain its vitality in biofertilizer. Observation of all streaked plates is shown by Figure 4.4.

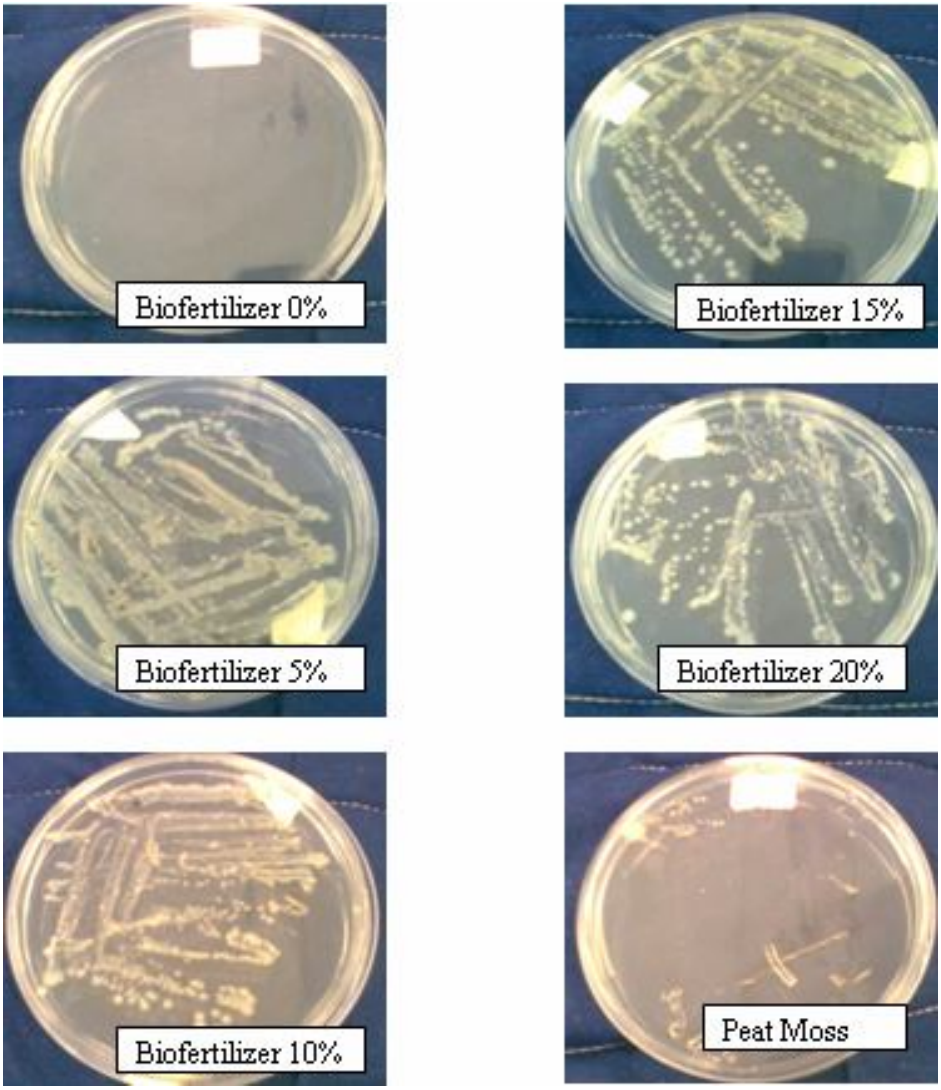

Figure 4.4: Testing on presence of N-fixer in biofertilizer after 14 days. No organism growth was observed on biofertilizer 0% and peat moss plates. Only N-fixer growths were observed on biofertilizer 5%, 10%, 15% and 20% plates.

4.2.3 Total Nitrogen Content in Biofertilizer

From the result of Kjeldahl method, it is clearly shown that nitrogen content was increasing with the increasing of N-fixer percentage. Although percentage of nitrogen are relatively small, accumulation of nitrogen content can be relate with

addition of N-fixer to biofertilizer from 5 to 20% v/w. Figure 4.5 shows the percentage of nitrogen content in biofertilizer after 14 days incubation with N-fixer.

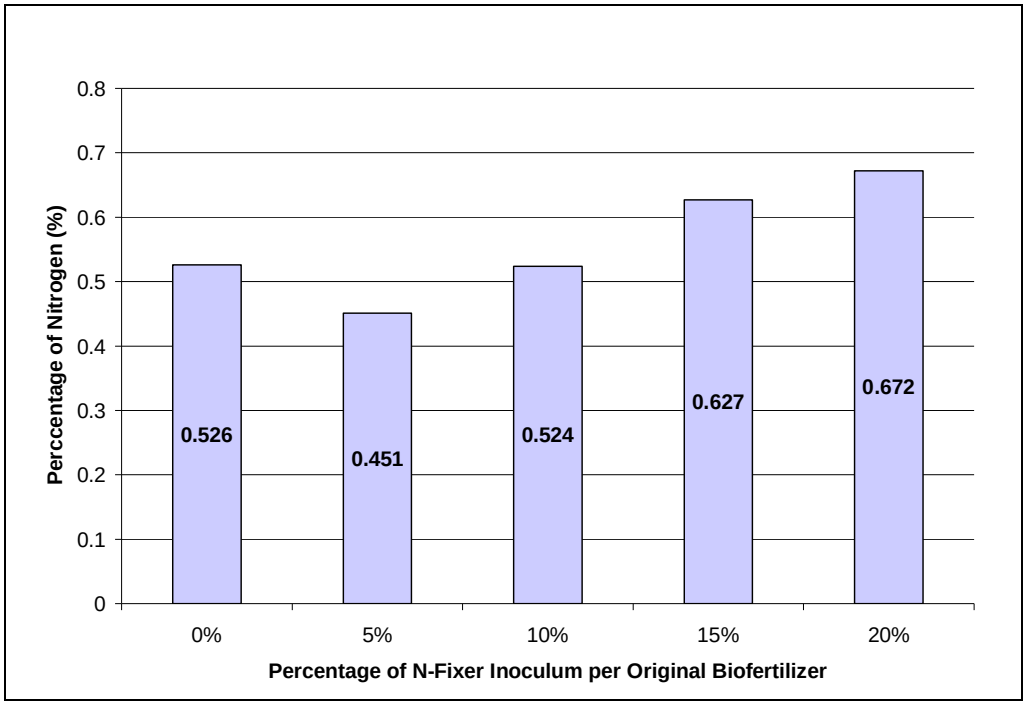

Figure 4.5: Percentage of N-fixer in biofertilizer after 14 days incubation

Enhancement of biofertilizer with 20% inoculum content, increased nitrogen content up to 27.7% compared to unmodified biofertilizer. These increments depicted that N-fixer still viable after 14 days incubation in biofertilizer. Biofertilizer 5%, have the lowest percentage of nitrogen content. This might due to low population of N-fixer to accumulate mineral nitrogen. However, nitrogen content in biofertilizer 0% was somewhat higher than biofertilizer 5%. It is believed that N-fixer use up some of peat moss while *Mycorrhiza* and *Trichoderma* did not use up peat moss as substrate and this maintain nitrogen content. When microbes are using peat moss as substrate, nitrogen content in sample is decreased. This is because, compost (peat moss) increases quantity and quality of total organic carbon, N and P nutrients (Melero *et al.*, 2007) if it is added to biofertilizer or soil. It is concluded here that addition of N-fixer percentage inoculum lower than 10% does not give benefit in term of additional nutrient.

4.3 Physical Observation of Patchouli

4.3.1 Number of Leaves

Recorded data on total changes of leaves were plotted against time to observe effect of different treatment to growth progress. Figure 4.6 shows the average patchouli's leaves growth progress.

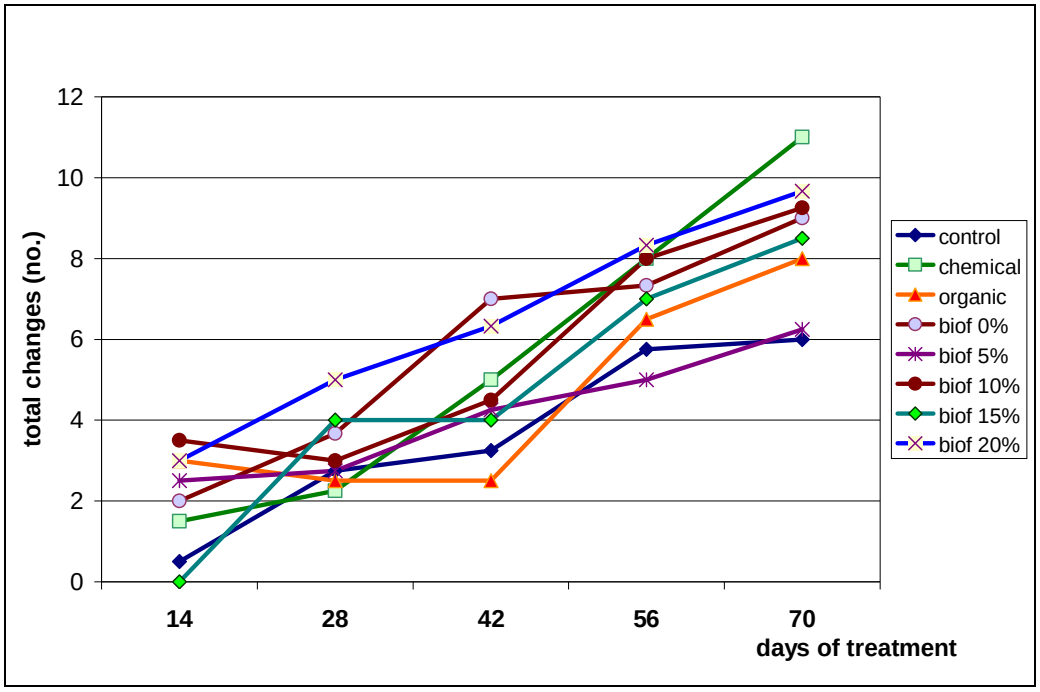

Figure 4.6: Total leaves changes over 70 days of plantation

Referring to Figure 4.6, all patchouli plant experienced leaves development over time. Treatment by chemical fertilizer shows highest total leaves changes followed by biofertilizer with 20% and 10% of N-fixer. Treatment by biofertilizer 5% and organics fertilizer to patchouli show the slowest development over time. This might due to low population of N-fixer in biofertilizer 5% to supply additional nutrient and lower nutrient content available in organics fertilizer. It is concluded that for leaves development, enhanced biofertilizer can give better effect if sufficient amount of N-fixer strain is added.

4.3.2 Number of Branches

Recorded data on total changes of branch were plotted against time to observe the effect of different treatment to growth progress. Figure 4.7 shows average patchouli's branch growth progress.

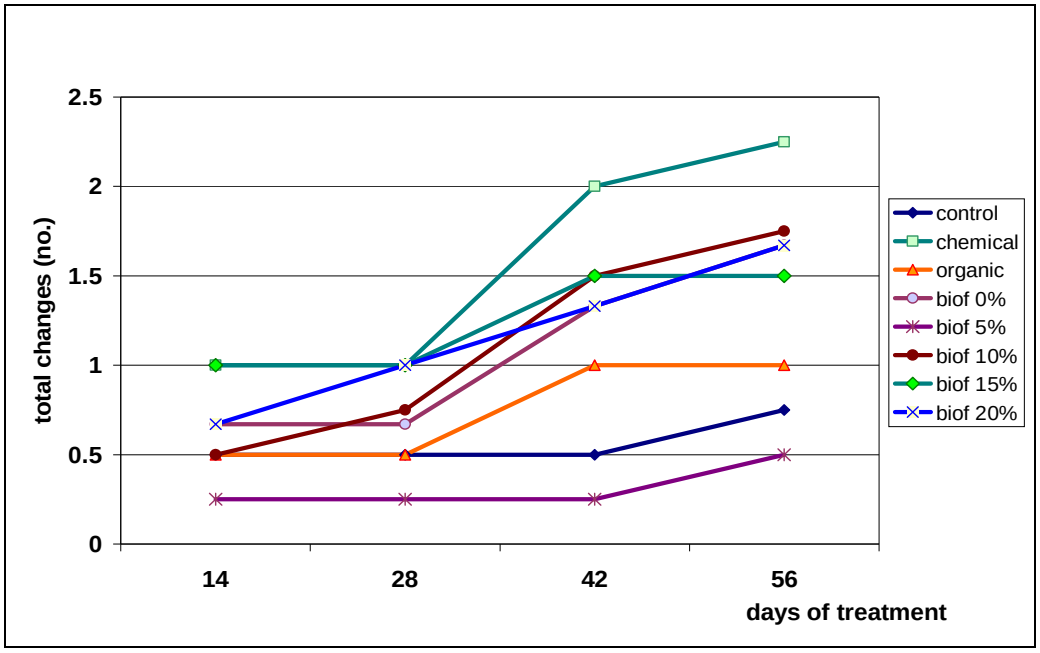

Figure 4.7: Total branches changes over 70 days of plantation

Based on Figure 4.7, it is shown that patchouli treated with biofertilizer 5% and organics fertilizer experienced the lowest branch development. It is believed that low population of N-fixer in biofertilizer 5% failed to cooperate with *Mycorrhiza* and *Trichoderma* to supply essential nutrient for plant. The biggest branch development is experienced by patchouli plant that using chemical fertilizer followed by biofertilizer 10%, biofertilizer 0%, biofertilizer 20% and biofertilizer 15%. It is concluded that using enhanced biofertilizer with 10 to 20% v/w of N-fixer give good effect to branch development of patchouli plant.

4.3.3 Height

Same graph were plotted for average plant's height total changes. The graph is shown by Figure 4.8.

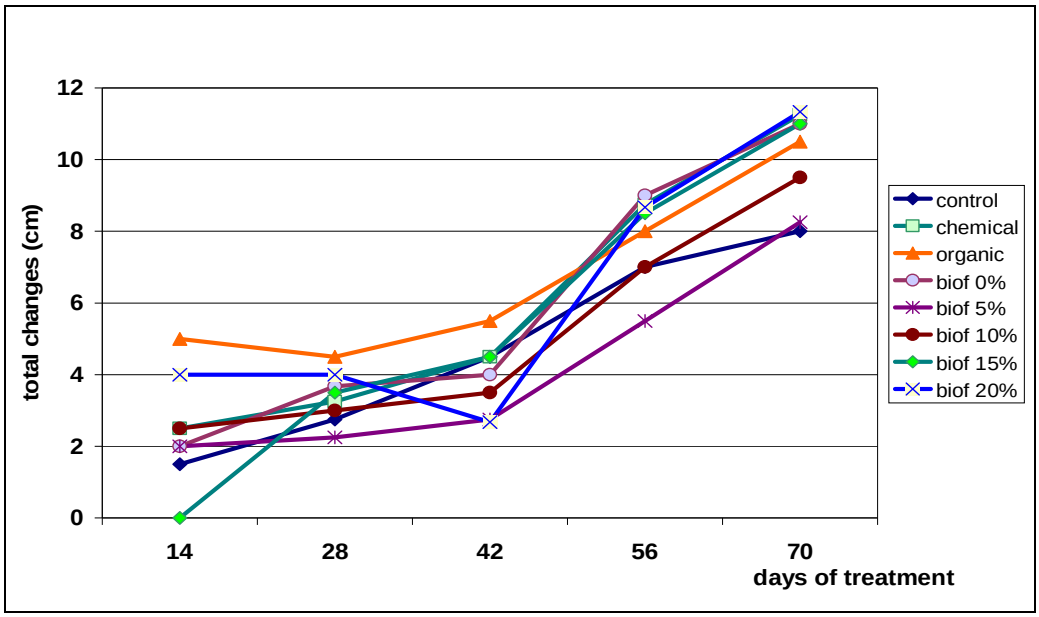

Figure 4.8: Total height changes over 70 days of plantation

For height of plant changes, biofertilizer with addition of 20% N-fixer inoculum give better effect compared to others treatment. This followed by chemical fertilizer and unmodified biofertilizer. It is shown here that unmodified biofertilizer give effect as good as chemical fertilizer and are better compared to some modified biofertilizer. Further study to enhance biofertilizer effect to patchouli height should be done. The height changes were not as important as leaves and branch development because patchouli oil is extracted from its leaves and branches. It is significant for patchouli propagation from the stems.

4.3.4 Summary of Physical Observation

In order to obtain overall observation, all physical observation was plotted in one graph. Averages readings were obtained by accumulated and divided the data by the total samples. The summary of total changes is shown by Figure 4.9.

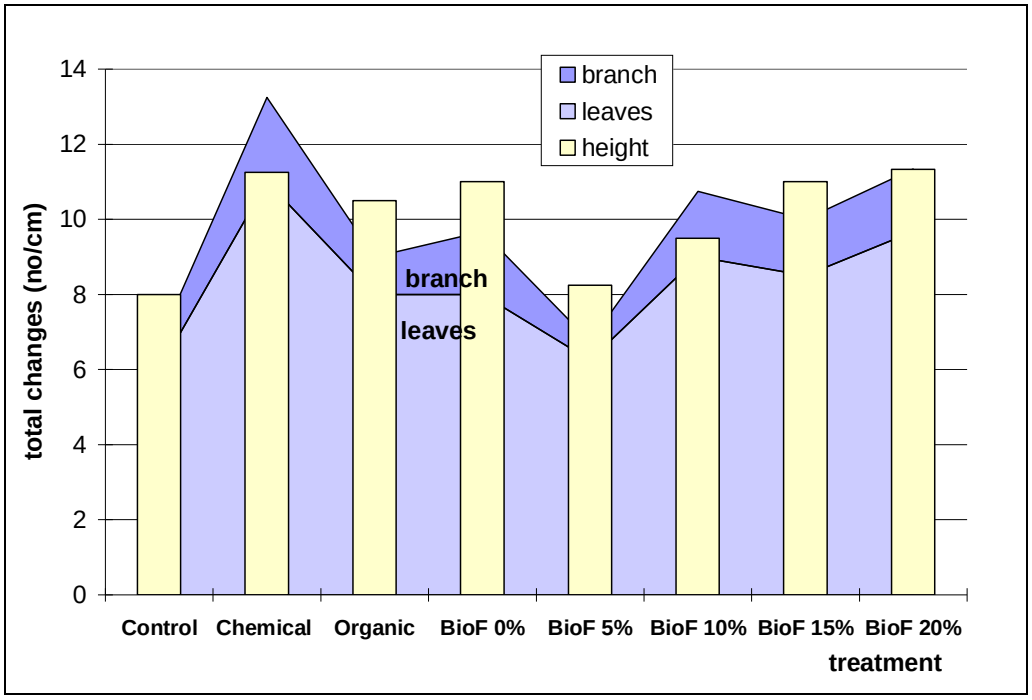

Figure 4.9: Summary of total changes

Generally, patchouli plant with enhanced biofertilizer experience more growth compared to original fertilizer (control). For overall leaves changes, treatment with chemical fertilizer give the biggest changes up to 83.3% enhancement compare to control. This followed by biofertilizer 20% (61.2%) and 10% (50.0%). NIIR Board (2004) has stated that in general, increase in yield (based on physical changes) due to biofertilizer application was of the order of 15 to 20%. Effect of the treatment to branch development was poorly recorded since the total changes are too small due to short duration of treatment. Treatments by chemical fertilizer experience the highest changes of branches development up to 200.0%. This followed by biofertilizer 10%, 20% and 0%, and 15% which experience 133.3%, 122.7% and 100.0% respectively. Lastly, the largest height of plant changes are experience by

treatment of biofertilizer 20% which is 41.6 % compared to control. This is followed by chemical fertilizer (40.6%), biofertilizer 15% (37.5%) and biofertilizer 0% (37.5%).

It can be observed that chemical fertilizer is able to provide superior effect to patchouli plant compare to others fertilizers. Even though the modified biofertilizer was unable to compete with original biofertilizer for height of plant changes, it was proved that modified biofertilizer 10% to 20% can increased the number of branch and leaves compared to organic and unmodified biofertilizer. Application of enhanced biofertilizer on patchouli showed improvement of leaves and branch growth up to 8% and 5% compared to original biofertilizer.

Original biofertilizer contains *Mycorrhiza* and *Trichoderma* where *Mycorrhiza* has high potential in accumulating phosphorus in the plants (NIIR Board. 2004) and helps to absorb and dissolve other nutrients for plants by storage in the root it is associated with (FNCA Biofertilizer Project Group, 2006). Specific research of *Mycorrhiza* by Arpana *et al.,* (2008) found that using *Mycorrhiza* will resulted in significant increase in plant height, number of branches, plant spread, plant biomass and P content in patchouli plant.

It can be concluded that additional nitrogen supply by N-fixer helps in development of patchouli plants. This fit with the statement of Bhaskar (1995) claims that application of nitrogen fertilizers will increase the productivity of patchouli. Others studies also shows that nitrogen fertilizer will increase the yield of herbage such as Patchouli (Singh and Rao, 2008). Moreover, biofertilizer application is suitable for non-legumes plants such as patchouli and this was supported by Doebereiner and Pedrosa (1987) whose claims that the effect of nitrogen fixation induced by nitrogen fixers is not only significant for legumes, but also for non-legumes.

All in all, using biofertilizer in plant growth will enhance the yield as good as using chemical fertilizer. Some improvement is still needed such as addition of

others strains that can enhance the supply of essential nutrient to plants such as potassium and trace elements. Complex biofertilizers contains different strain have been reported to be more effective in increasing nutrient supply and crop yield (NIIR Board, 2004).

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CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

From growth profile, it is concluded that highest yield of active N-fixer can be obtained at 16 hours of fermentation at condition 29^{o}C and 200 rpm using initial glucose concentration 10 g/L and initial pH 7.4. Increasing N-fixer population does increase nitrogen content in biofertilizer. There are significance improvements of plants growth after using enhanced biofertilizer that contained N-fixer. It is concluded that additional nitrogen supply by N-fixer helps in development of patchouli plants. Application of enhanced biofertilizer on patchouli showed improvement of leaves and branch growth up to 8% and 5% respectively compared to original biofertilizer. Biofertilizer can also offer yield as good as using chemical fertilizer if complex biofertilizer containing different strain that supply different nutrient are used. The suitable range of inoculum should be applied to biofertilizer is more than 10%.

5.2 Recommendation

In order to improve this research, there are several things should be stress out in the future. It is proposed to make some changes for Kjeldahl method or automated Total Kjeldahl Nitrogen Equipment should be provided.

Relationship of N-fixer, *Trichoderma* and *Mycorrhiza* should be analyzed further to ensure that they are not competitive with each others.

Total number of patchouli plant used in this study should be increased while initial plants used and soil properties should be more standardized. In order to increase percentage of patchouli plant survived until the end of experiment, older stems cut should be used for propagation. Patchouli should age more than 45 days before undergoes fertilizers treatment. In this study, the patchouli used is age 14 to 60 days. Furthermore, larger poly bag should be considered to avoid accumulation of nutrient near roots that cause patchouli sag and died. All of this will increase the accuracy of experiments result.

For further study, it is recommended that experiment is continued until downstream oil extraction and purification. Patchouli oil can be extracted from plants and oil content can be analyzed by gas chromatography-mass spectrometry (GCMS) to observe the effect of enhanced biofertilizer to patchouli oil composition.

Since growth profile is already known, some steps can be eliminated and time for treating patchouli can be increased from 10 weeks to 16 weeks (4 month). By doing this, effect of different treatment can be seen well.

Enhancement of biofertilizer can be continued by combining the present organism (N-fixer, *Mycorrhiza* and *Trichoderma*) with other effective strain such as potassium solubilizer bacteria and organic acid bacteria. This will overcome the problem due to limited types of nutrient can be supplied by a biofertilizer to plants.

Percentage of N-fixer inoculum applied to biofertilizer can be increase to more than 20% v/w.

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APPENDIX A

COMPOSITION OF GROWTH MEDIUM

A1 Composition of Nutrient Agar

Ingredients	Gms/litre
Peptic Digest of Animal Tissue	5.00
Beef Extract	1.50
Yeast Extract	1.50
Sodium Chloride	15.00

Nutrient Agar (NA) is prepared by dissolving 28 g of instant nutrient agar into 1000 mL of distilled water. Next, it is heated until boiled. Then, it is autoclaved at 6.80 kg pressure (121^{o}C) for 15 minutes. After cooled it for a little bit, it is poured into container (test tube, slant bottle, agar plate etc.). Final pH at 25^{o}C is 7.4+-0.2.

A2 Composition of Nutrient Broth

Ingredients	Gms/litre
Peptic Digest of Animal Tissue	5.00
Beef Extract	1.50
Yeast Extract	1.50
Sodium Chloride	5.00

Nutrient Broth (NB) is prepared by dissolving 13.00 g of instant nutrient broth into 1000mL of distilled water. Heat is supply only if the powder does not completely dissolve in distilled water. Then, it is autoclaved at 6.80 kg pressure (121^{o}C) for 15 minutes and cooled before it is ready to be used. Final pH at 25^{o}C is 7.4+-0.2.

APPENDIX B

GLUCOSE ASSAY (Miller 1959)

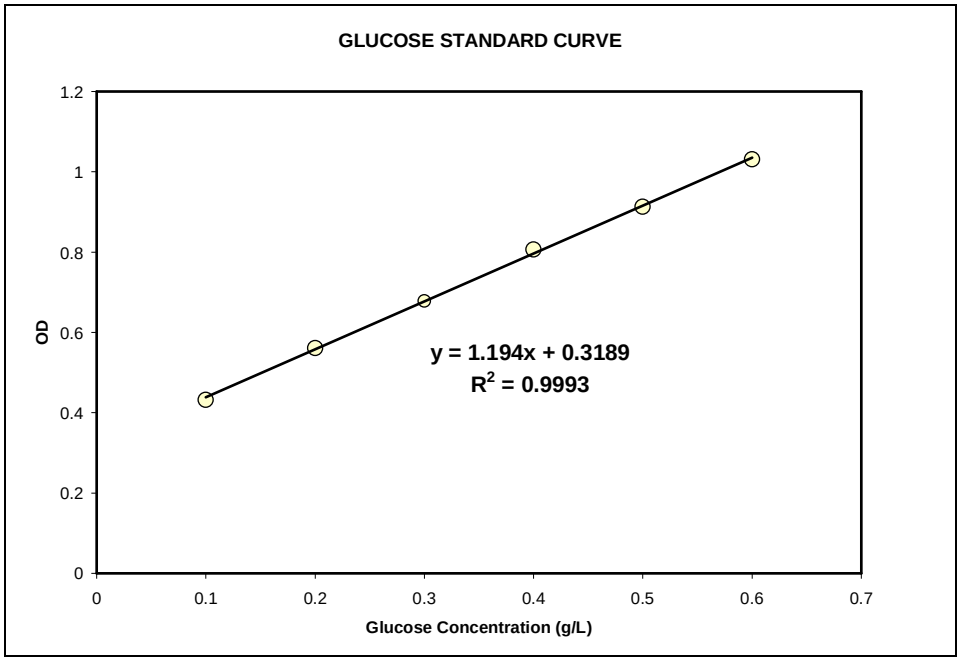
B1 DNS Reagent Preparation

10 g NaOH
182 g Sodium Potassium Tartarate
2 g Phenol
0.5 g Sodium Sulphite
10 g Dinitrosalicylic acid

The entire ingredient is dissolved in 600mL distilled water before top up to 1 liter in amber bottle or cover with aluminium foil. Then it is stir overnight. It is keep at 4^{o}C in refrigerator.

B2 Glucose Standard Curve

Concentration (g/L)	0	0.1	0.2	0.3	0.4	0.5	0.6
OD	0	0.431	0.562	0.678	0.806	0.913	1.030



APPENDIX C

FERMENTATION PROFILE OF N-FIXER

C1 Optical Density Reading

Table C1: Optical density of N-fixer

Time (hour)	Shake flask 1			Shake flask 2		
	Read 1	Read 2	Average	Read 1	Read 2	Average
0	0.311	0.312	0.312	0.285	0.280	0.283
3	0.463	0.464	0.464	0.427	0.425	0.426
6	1.247	1.251	1.249	1.058	1.110	1.084
8	1.442	1.451	1.447	1.454	1.459	1.457
12	1.715	1.728	1.722	1.585	1.656	1.621
13	1.760	1.775	1.768	1.724	1.715	1.720
21	2.201	2.229	2.215	2.215	2.222	2.219
25	2.284	2.252	2.268	2.237	2.244	2.241
28	2.347	2.357	2.352	2.301	2.293	2.297
31	2.377	2.409	2.393	2.377	2.377	2.377
34	2.456	2.456	2.456	2.398	2.366	2.382
37	2.398	2.398	2.398	2.366	2.377	2.372
46	2.537	2.537	2.537	2.482	2.456	2.469
52	2.509	2.482	2.500	2.432	2.456	2.444
56	2.523	2.523	2.523	2.468	2.482	2.475
60	2.482	2.482	2.482	2.409	2.409	2.409

C2 Glucose Concentration

$$y = 1.194x + 0.3189$$

$$\text{Glucose Concentration} = [(\text{OD} \times 10) - 0.3189]/1.194$$

Table C2: Glucose density and concentration

Time (hour)	Replicate 1		Replicate 2	
	OD glucose	[Glucose] (d=1)	OD glucose	[Glucose] (d=1)
0	0.681	4.81	0.745	5.26
3	0.700	4.94	0.747	5.27
6	0.753	5.32	0.723	5.10
8	0.700	4.94	0.707	4.99
12	0.646	4.56	0.600	4.24
13	0.708	5.00	0.638	4.50
21	0.648	4.57	0.645	4.55
25	0.642	4.53	0.594	4.19
28	0.550	3.88	0.583	4.12
31	0.532	3.76	0.555	3.92
34	0.510	3.60	0.462	3.26
37	0.486	3.43	0.534	3.77
46	0.319	2.25	0.348	2.46
52	0.351	2.48	0.424	2.99
56	0.322	2.27	0.303	2.14
60	0.267	1.89	0.314	2.22

APPENDIX D

NITROGEN CONTENT BY KJELDAHL METHOD

D1 Volume NaOH used for Titration

Table D1: Volume of NaOH and percentage of nitrogen

Biofertilizer	Volume of 0.1 **N** NaOH, used for Titration, mL					
		1	2	3	4	Acceptable %
0%	Volume	6.2	8.8	9.0	11.7	0.526
	%	0.680	0.608	0.602	0.526	
5%	Volume	14.0	14.4	-	-	0.451
	%	0.462	0.451	-	-	
10%	Volume	7.8	11.8	-	-	0.524
	%	0.636	0.524	-	-	
15%	Volume	8.8	8.1	-	-	0.627
	%	0.608	0.627	-	-	
20%	Volume	-	6.5	-	-	0.672
	%	[-]tive	0.672	-	-	

*Volume of 0.1 **N** NaOH used for blank = 30.5 mL

Percentage of Nitrogen = ($blank_{mL}$ – volume NaOH) x 0.028

- Biofertilizer 0% = (30.5_{mL} – 11.7_{mL}) x 0.028 = 0.526 %
- Biofertilizer 5% = (30.5_{mL} – 14.4_{mL}) x 0.028 = 0.451 %
- Biofertilizer 10% = (30.5_{mL} – 11.8_{mL}) x 0.028 = 0.524 %
- Biofertilizer 15% = (30.5_{mL} – 8.1_{mL}) x 0.028 = 0.627 %
- Biofertilizer 20% = (30.5_{mL} – 6.5_{mL}) x 0.028 = 0.672 %

APPENDIX E

OBSERVATION ON PATCHOULI

E1 Set A

Table E1: Observation of set A

Parameter	Total Changes (cm)																	
	3/12			17/12			31/12			14/01			28/01			14/02		
Control	12	2	13	13	2	14	16	2	17	16	2	19	18	2	24	13	3	22
Chemical	22	3	17	25	5	20	31	6	20	29	5	22	30	6	24	32	6	25
Organic	16	3	8	19	3	13	21	4	15	21	4	17	27	5	21	29	5	23
Biofertilizer 0%	15	3	13	17	4	15	20	4	17	26	4	18	27	5	22	28	5	22
Biofertilizer 5%	9	2	18	13	3	22	15	3	23	19	3	24	20	3	30	21	3	34
Biofertilizer 10%	14	2	23	18	3	26	23	5	30	27	5	26	36	7	36	33	7	37
Biofertilizer 15%	18	3	17	18	3	17	24	5	21	24	5	22	28	6	26	32	6	29
Biofertilizer 20%	11	3	22	17	4	28	24	5	29	25	6	31	31	6	37	33	6	39

Physical observation: 1st column = number of leaves)(2nd column = number of branch)(3rd column = patchouli height)

Table E2: Total changes of set A

Physical Observation	Treatment	Total Changes (cm)				
		17/12	31/12	14/01	28/01	14/02
Leaves	Control	1	4	4	6	6
	Chemical	3	5	7	8	10
	Organic	3	5	5	11	13
	Biofertilizer 0%	2	5	11	12	13
	Biofertilizer 5%	4	6	10	11	13
	Biofertilizer 10%	4	9	13	22	19
	Biofertilizer 15%	0	6	6	10	14
	Biofertilizer 20%	6	13	14	20	22
Branch	Control	0	0	0	0	1
	Chemical	2	2	2	3	3
	Organic	0	1	1	2	2
	Biofertilizer 0%	1	1	1	2	2
	Biofertilizer 5%	1	1	1	1	1
	Biofertilizer 10%	1	2	3	5	5
	Biofertilizer 15%	0	2	2	3	3
	Biofertilizer 20%	1	2	3	3	3
Height	Control	1	4	7	8	9
	Chemical	3	3	5	7	8
	Organic	5	7	9	13	15
	Biofertilizer 0%	2	4	5	9	9
	Biofertilizer 5%	4	5	6	12	16
	Biofertilizer 10%	3	7	7	13	14
	Biofertilizer 15%	0	4	5	9	12
	Biofertilizer 20%	6	7	9	15	17

E2 Set B

Table E3: Observation of set B

Parameter	Total Changes (cm)																	
	3/12			17/12			31/12			14/01			28/01			14/02		
Control	13	2	8	13	2	10	20	4	15	19	4	16	23	4	19	23	4	20
Chemical	7	2	9	7	3	11	14	4	17	18	4	19	23	5	26	29	6	31
Organic	9	2	8	17	2	8	12	2	8	7	2	8	D	D	D	D	D	D
Biofertilizer 0%	9	2	11	11	3	13	13	3	16	17	3	16	16	4	23	20	4	26
Biofertilizer 5%	12	2	9	13	2	9	16	2	10	15	2	10	17	2	12	16	2	12
Biofertilizer 10%	4	2	2	7	2	4	6	2	4	6	2	4	9	2	5	12	2	6
Biofertilizer 15%	4	1	6	4	1	7	4	1	7	6	1	7	6	1	9	D	D	D
Biofertilizer 20%	10	2	11	10	2	13	12	2	14	14	2	15	13	2	16	14	2	19

*D = patchouli dead

Table E4: Total changes of set B

Physical Observation	Treatment	Total Changes (cm)				
		17/12	31/12	14/01	28/01	14/02
Leaves	Control	0	7	6	10	10
	Chemical	0	7	11	16	22
	Organic	D	D	D	D	D
	Biofertilizer 0%	2	4	8	7	11
	Biofertilizer 5%	1	4	3	5	4
	Biofertilizer 10%	3	2	2	5	8
	Biofertilizer 15%	D	D	D	D	D
	Biofertilizer 20%	0	2	4	3	4
Branch	Control	0	2	2	2	2
	Chemical	1	2	2	3	4
	Organic	D	D	D	D	D
	Biofertilizer 0%	1	1	1	2	2
	Biofertilizer 5%	0	0	0	0	0
	Biofertilizer 10%	0	0	0	0	0
	Biofertilizer 15%	D	D	D	D	D
	Biofertilizer 20%	0	0	0	0	0
Height	Control	2	7	8	11	12
	Chemical	2	8	10	17	22
	Organic	D	D	D	D	D
	Biofertilizer 0%	2	5	5	12	15
	Biofertilizer 5%	0	1	1	3	3
	Biofertilizer 10%	2	2	2	3	4
	Biofertilizer 15%	D	D	D	D	D
	Biofertilizer 20%	2	3	4	5	8

E3 Set C

Table E5: Observation of set C

Parameter	Total Changes (cm)														
	17/12			31/12			14/01			28/01			14/02		
Control	4	1	6	4	1	8	6	1	8	7	1	12	8	1	14
Chemical	4	1	8	4	1	10	4	1	10	6	2	14	6	2	14
Organic	4	1	6	4	1	8	4	1	8	6	1	9	7	1	12
Biofertilizer 0%	3	1	10	5	1	12	5	1	12	6	1	16	6	2	19
Biofertilizer 5%	4	1	12	4	1	14	4	1	15	6	1	17	8	2	21
Biofertilizer 10%	5	1	10	5	1	12	6	1	14	6	2	19	9	3	24
Biofertilizer 15%	4	1	10	6	1	13	6	1	14	8	1	18	7	1	20
Biofertilizer 20%	5	1	12	5	1	14	6	1	15	7	2	18	8	3	21

Table E6: Total changes of set C

Physical Observation	Treatment	Total Changes (cm)			
		31/12	14/01	28/01	14/02
Leaves	Control	0	2	3	4
	Chemical	0	0	2	2
	Organic	0	0	2	3
	Biofertilizer 0%	2	2	3	3
	Biofertilizer 5%	0	0	2	4
	Biofertilizer 10%	0	1	1	4
	Biofertilizer 15%	2	2	4	3
	Biofertilizer 20%	0	1	2	3
Branch	Control	0	0	0	0
	Chemical	0	0	1	1
	Organic	0	0	0	0
	Biofertilizer 0%	0	0	0	1
	Biofertilizer 5%	0	0	0	1
	Biofertilizer 10%	0	0	1	2
	Biofertilizer 15%	0	0	0	0
	Biofertilizer 20%	0	0	1	2
Height	Control	2	2	6	8
	Chemical	2	2	6	6
	Organic	2	2	3	6
	Biofertilizer 0%	2	2	6	9
	Biofertilizer 5%	2	3	5	9
	Biofertilizer 10%	2	4	9	14
	Biofertilizer 15%	3	4	8	10
	Biofertilizer 20%	2	3	6	9

E4 Set D

Table E7: Observation of set D

Parameter	Total Changes (cm)														
	17/12			31/12			14/01			28/01			14/02		
Control	4	2	9	4	2	9	5	2	10	8	2	12	8	2	12
Chemical	2	1	1	2	1	1	4	1	2	8	2	6	12	2	10
Organic	3	1	6	3	1	7	2	1	7	D	D	D	D	D	D
Biofertilizer 0%	2	1	1	2	1	2	D	D	D	D	D	D	D	D	D
Biofertilizer 5%	2	1	7	3	1	8	6	1	8	4	1	9	6	1	12
Biofertilizer 10%	2	1	5	3	1	6	4	1	6	6	1	8	8	1	11
Biofertilizer 15%	4	1	3	3	1	4	2	1	4	D	D	D	D	D	D
Biofertilizer 20%	4	1	1	2	1	2	D	D	D	D	D	D	D	D	D

Table E8: Total changes of set D

Physical Observation	Treatment	Total Changes (cm)			
		31/12	14/01	28/01	14/02
Leaves	Control	0	1	4	4
	Chemical	0	2	6	10
	Organic	D	D	D	D
	Biofertilizer 0%	D	D	D	D
	Biofertilizer 5%	1	4	2	4
	Biofertilizer 10%	1	2	4	6
	Biofertilizer 15%	D	D	D	D
	Biofertilizer 20%	D	D	D	D
Branch	Control	0	0	0	0
	Chemical	0	0	1	1
	Organic	D	D	D	D
	Biofertilizer 0%	D	D	D	D
	Biofertilizer 5%	0	0	0	0
	Biofertilizer 10%	0	0	0	0
	Biofertilizer 15%	D	D	D	D
	Biofertilizer 20%	D	D	D	D
Height	Control	0	1	3	3
	Chemical	0	1	5	9
	Organic	D	D	D	D
	Biofertilizer 0%	D	D	D	D
	Biofertilizer 5%	1	1	2	5
	Biofertilizer 10%	1	1	3	6
	Biofertilizer 15%	D	D	D	D
	Biofertilizer 20%	D	D	D	D

E5 Average Physical Observation

Table E9: Average physical observation

	Treatment	**17/12**	**31/12**	**14/01**	**28/01**	**14/02**
	control	0.5	2.8	3.3	5.8	6.0
	chemical	1.5	2.3	5.0	8.0	11.0
	organic	3.0	2.5	2.5	6.5	8.0
	Biofertilizer 0%	2.0	3.7	7.0	7.3	9.0
	Biofertilizer 5%	2.5	2.8	4.3	5.0	6.3
	Biofertilizer 10%	3.5	3.0	4.5	8.0	9.3
	Biofertilizer 15%	0.0	4.0	4.0	7.0	8.5
Leaves	Biofertilizer 20%	3.0	5.0	6.3	8.3	9.7
	Treatment	**17/12**	**31/12**	**14/01**	**28/01**	**14/02**
	control	0.0	0.5	0.5	0.5	0.8
	chemical	1.5	1.0	1.0	2.0	2.3
	organic	0.0	0.5	0.5	1.0	1.0
	Biofertilizer 0%	1.0	0.7	0.7	1.3	1.7
	Biofertilizer 5%	0.5	0.3	0.3	0.3	0.5
	Biofertilizer 10%	0.5	0.5	0.8	1.5	1.8
	Biofertilizer 15%	0.0	1.0	1.0	1.5	1.5
Branch	Biofertilizer 20%	0.5	0.67	1.0	1.3	1.7
	Treatment	**17/12**	**31/12**	**14/01**	**28/01**	**14/02**
	control	1.5	2.75	4.5	7.0	8.0
	chemical	2.5	3.25	4.5	8.75	11.25
	organic	5.0	4.5	5.5	8.0	10.5
	Biofertilizer 0%	2.0	3.67	4.0	9.0	11.0
	Biofertilizer 5%	2.0	2.25	2.75	5.5	8.25
	Biofertilizer 10%	2.5	3.0	3.5	7.0	9.5
	Biofertilizer 15%	0.0	3.5	4.5	8.5	11.0
Height	Biofertilizer 20%	4.0	4.0	2.67	8.67	11.33

E6 Patchouli Plants

Figure E1: Patchouli (initial Set A and B)

Figure E2: Patchouli (initial Set C and D)

Figure E3: Patchouli (Set A, B, C and D at the end of experiment)

APPENDIX F

FERTILIZER USED AND COMPONENT

F1 Chemical Fertilizer

Brand	:Nitrophoska Green
Made from	:Chemical routes
Component	:N=15%, P=15%, K=15%
Produced in	:Belgium
Quantity used	:10 granules per poly bag per every two week

F2 Organic Fertilizer

Brand	:Saby Baja Organik
Made from	:Cow dung
Component	:N=2%, P=0.65%, K=1.95%, Mg=0.55%, Ca=1.25%
Produced by	:SABY Enterprise, Kg Bukit Bangkong, 43900 Sepang
Quantity used	:100 gram per poly bag per every two week

F3 Biofertilizer

Brand	:Makmur Bio-Organik
Made from	:Palm oil remains with Mycorrizae and Tricoderma Reesei
Component	:N=2.5%, P=2.5%, K=3%, and 8 trace element
Produced by	:Dominion Media Sdn Bhd, Lepar Hilir, Pahang
Quantity used	:100 gram per poly bag per every two week

F4 Modified Biofertilizer

0%	:1200 gram Biofertilizer, 150 gram peat moss
5%	:1200 gram Biofertilizer, 150 gram peat moss, 60 mL N-fixer
10%	:1200 gram Biofertilizer, 150 gram peat moss, 120 mL N-fixer

15% :1200 gram Biofertilizer, 150 gram peat moss, 180 mL N-fixer

20% :1200 gram Biofertilizer, 150 gram peat moss, 240 mL N-fixer