GROWTH OPTIMIZATION OF POTASSIUM SOLUBILIZING BACTERIA ISOLATED FROM BIOFERTILIZER

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ABSTRACT

Potassium is one of the essential nutrients for plant to growth and required in large amount to achieve optimum growth. Biofertilizer is a good platform to deliver this primary macronutrient by assistance of Potassium Solubilizing Bacteria (KSB). To acquire the optimum outcome by applying this bacterium into the biofertilizer, the optimum growth condition must be studied. The optimum growth conditions for KSB based on temperature, pH and initial glucose concentration parameters were studied during this research. The Design of Experiment (DOE) software was used to design the optimization experimental work and analyse the results produced. The range for these three parameters were set at 25°C - 32°C, pH 6 - pH 8 and 8g/L - 13 g/L respectively. The fermentation was run for 72 hours at 200rpm of agitation rate in incubator shaker. Two responses were studied during fermentation process which were cell number and glucose consumption. The fermentation process was run in 250 mL shake flasks. Bacteria's cell concentration was determined by using colony forming unit (CFU) method. On the other hand, glucose consumption was measured by using DNS method. All the results were transferred into the DOE software for analysis process. Within the stated range, the software suggested that the optimum growth condition for Potassium Solubilizing Bacteria is at temperature of 32°C, pH 8 and with 8g/L initial glucose concentration.

ABSTRAK

Kalium merupakan salah satu nutrisi yang penting untuk pertumbuhan tumbuhan dan diperlukan dalam kuantiti yang besar bagi mencapai kadar pertumbuhan yang optimum. Baja organik merupakan satu kaedah yang amat baik bagi membekalkan makronutrien ini kepada tumbuhan dengan bantuan bakteria pelarut kalium (KSB). Untuk mencapai hasil dan kesan yang optimum dengan menggunakan KSB, keadaan pertumbuhan optimum KSB perlu dikaji. Keadaan optimum bagi pertumbuhan KSB berdasarkan parameter suhu, pH dan kepekatan awal glukosa telah dikaji. Perisian Design of Experiment (DOE) telah digunakan untuk mereka bentuk eksperimen dan menganalisis keputusan yang diperolehi bagi mencari keadaan optimum untuk pertumbuhan KSB. Julat bagi ketiga-tiga parameter tersebut ialah masing-masing 25°C - 32°C, pH 6 - pH 8 dan 8g/L - 13 g/L. KSB telah dieram selama 72 jam dengan kadar goncangan 200 rpm. Dua respon telah dikaji semasa proses fermentasi iaitu bilangan sel bakteria dan penggunaan glukosa. Proses fermentasi telah dijalankan di dalam kelalang 250 mL. Bilangan sel bakteria telah dihitung dengan menggunakan kaedah unit pembentukan koloni (CFU). Sementara itu, jumlah penggunaan glukosa pula dihitung dengan menggunakan kaedah DNS. Keputusan experimen yang telah diperolehi telah dimasukkan ke dalam perisian DOE untuk proses analisis. Dalam lingkungan julat yang telah dinyatakan, perisian tersebut telah mencadangkan bahawa keadaan optimum bagi pertumbuhan bakteria pelarut kalium adalah pada suhu 32°C, pH 8 dan pada kepekatan awal glukosa sebanyak 8 g/L.

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LIST OF SYMBOLS/ABBREVIATIONS

ATP	-	Adenosine triphosphate
CFU	-	Colony Forming Unit
DNA	-	Deoxy-ribonucleic acid
g	-	gram
g/L	-	gram per liter
HCl	-	hydrochloric acid
hr	-	hour
K^+	-	Potassium ion
KSB	-	Potassium Solubilizing Bacteria
LB	-	Luria-Bertani broth
ml	-	mililiter
NADP	-	Nicotinamide Adenine Nucleotide Phosphate
NaOH	-	Sodium hydroxide
NH_4^+	-	Ammonium
nm	-	nanometer
NO_3^-	-	Nitrate
NPK	-	Nitrogen, Phosphorus and Potassium
OD	-	Optical density
PGPR	-	Plant Growth Promoting Rhizobacteria
Rpm	-	Revolution per minute
v/v	-	volume per volume
°C	-	degree Celsius
°F	-	degree Fahrenheit
μL	-	microliter
%	-	percentage

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

In the last century, when the chemical fertilizers were first introduced into the agriculture field, most of the problems faced by farmers to increase yield of their plantation have been solve. However, chemical fertilizer slowly started to show their side effect on human and environment. After that, the use of biofertilizer was recommended to improve plant nutrient and production in sustainable way (Han *et al*. 2006).

Basically, biofertilizer is a substance which contains microorganisms that colonizes the rhizosphere or the zone that surrounds the roots of plants (Shen, 1997). These microorganisms have ability to convert nutritionally important elements such as nitrogen, phosphorus and potassium (NPK) from unavailable to available form through biological processes. There is evidence that prove the beneficial microbes had been used in agriculture practices since 60 years ago and now these beneficial microbial populations show that they can also resistance to adverse environmental stresses (Shen, 1997).

Potassium solubilizing bacteria (KSB) such as *Bacillus mucilagenosus* and *Bacillus edaphicus* are example of microorganisms that used in biofertilizer. KSB are able to solubilize potassium rock through production and secretion of organic acids (Han and Lee, 2005). Fundamentally, potassium is a macronutrient in plants and animals. Recent studies have proved that potassium can increase the plant height, fresh plant weight and also increase herbage and oil yield on the patchouli (Singh et *al*., 2008). However, during the last decades, the issue of sustainable soil potassium management has partly been ignored since the potential environment impact of nitrogen and phosphorus has been considered a more important problem. Furthermore, the application of biofertilizer somehow has not achieved constant effects compared to chemical fertilizer. The mechanisms and interactions among these microbes still are not well understood, especially in real applications. Therefore, the aim of this study to increase the bio-fertilizers effect on the crop growth by optimize the growth of KSB is very important.

1.2 Problems Statement

Biofertilizer give low quality effect on patchouli growth compared to chemical fertilizer in term of oil yield and growth condition. Longer time taken for nutrients supply by biofertilizer to the plants is one of the reason this situation occur compared to chemical fertilizer that can directly supply nutrients after mixing with soil. Nevertheless, the chemical fertilizer have side effect such as leaching out, polluting water basins, destroying microorganisms and friendly insects, and making the crop more susceptible to the attack of diseases. Thus, biofertilizer is more favorable and encouraging to be used for it eco-friendly. Therefore, to increase the quality of biofertilizer at least as good as chemical fertilizer, optimization of KSB growth which is used in biofertilizer is necessary to increase potassium uptake on plants.

1.4 Statement of Objectives

The aim of this study/research is to determine the optimum growth conditions of potassium solubilizing bacteria isolated from biofertilizer. Hence, the objectives of this research are:

a. To obtain the optimum temperature on the potassium solubilizing bacteria growth within stated range.
b. To obtain the optimum pH on the potassium solubilizing bacteria growth within stated range.
c. To obtain the optimum initial glucose concentration on the potassium solubilizing bacteria growth within stated range.

1.5 Scope of Study

To achieve the objectives of the research study, the bacteria growth curve with glucose consumption should be plotted first with other standard curves which are standard curve of glucose and optical density (OD) versus colony forming unit (CFU) curve. These standard curves are prepared during 72 hours of fermentation process and the data will be taken in 3 hours interval time. After standard curves had been prepared, growth optimization of KSB experiment will be started with generate series of lab work by using Design of Experiment software. This series of lab work are based on the range of the three parameters which are temperature (25°C to 32°C), pH (6 to 7.5) and initial glucose concentration (8 to 13 g/L). In the end, at certain combination of these three parameters values, the highest biomass of the KSB will be produced. At these parameters values, it is known as the optimum condition for KSB to growth.

CHAPTER 2

LITERATURE REVIEW

2.1 Important of Nitrogen, Phosphate and Potassium (NPK) to the Plant

Nutrients are essential for a plant to growth healthy. There are about 13 mineral nutrients in the soil and they are classified into two categories which are macronutrient and micronutrient depending on the quantity required (Lack and Evans, 2005). NPK are primary macronutrients element which are needed in large amounts while copper, boron and iron are example of micronutrients that are needed in only very small amount or micro quantity. For this reason, plants needs the right combination of nutrients to live, growth and reproduce beside avoid suffer from malnutrition. In the soil, the mineral nutrients are dissolved in water and absorbed through a plant's root. However, the amounts of nutrients in the soil are always unpredictable and not enough for plants growth. As a result, primary nutrients NPK which are utilized in the large amounts by crops are commonly found in blended fertilizers nowadays (Tucker, 1999).

2.1.1 Nitrogen

Based on the primary macronutrients NPK, plants required nitrogen in the largest amounts compared to phosphorus and potassium. However, unlike carbon dioxide and oxygen, the element form of nitrogen is completely unavailable to use directly to the majority of green plants (Hillel, 2008). Plants such as legumes gain nitrogen by associated with free living microorganisms which can fix the nitrogen from the atmosphere and introduce into biosphere. Besides, decaying organic materials, animal excreta and nitrogen fertilizer also contribute as source of nitrogen in the soil (Lack and Evans, 2005). Figure 2.1 show the nitrogen's cycle in soil and environment. Basically, nitrogen is taken up by plants in the form of either nitrate (NO_3^-) or ammonium (NH_4^+). Ammonium is produced by decomposers and nitrogen-fixing soil bacteria through ammonification process. Then, nitrifying bacteria will convert ammonium to nitrites as intermediate through nitrification process before producing nitrate that will take up by plant in assimilation process.

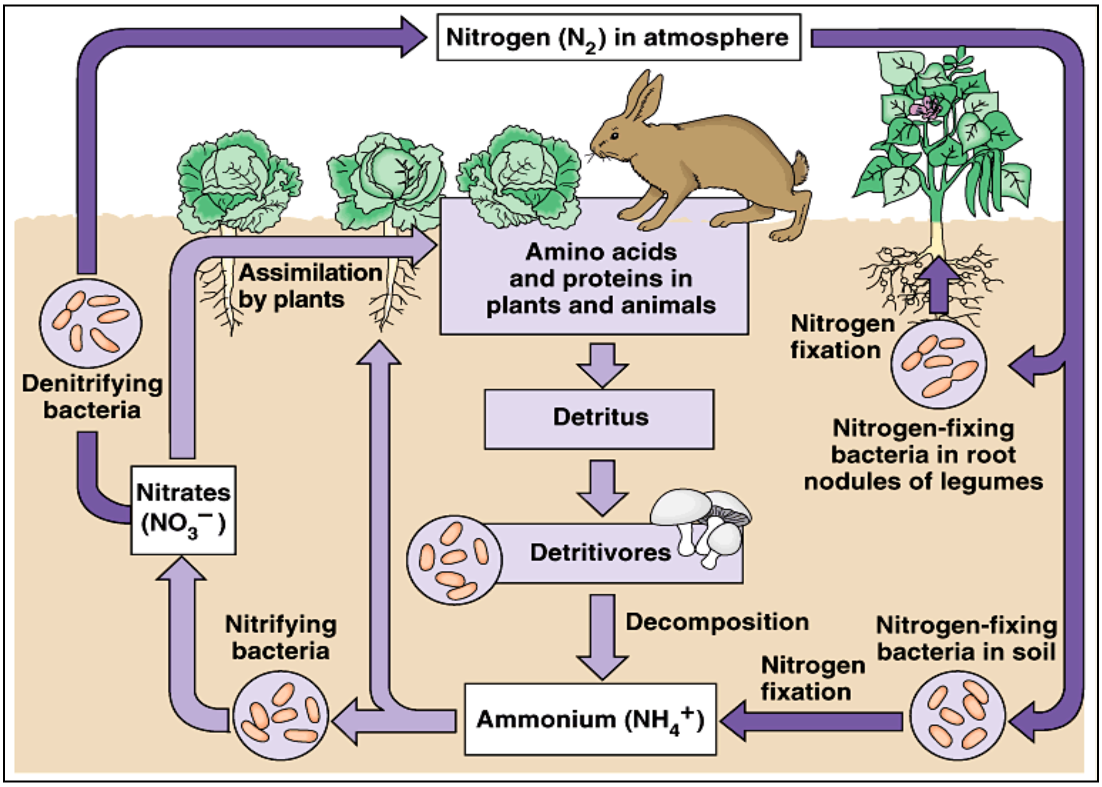

Figure 2.1: Nitrogen's Cycle. (Spiro and Stigliani, 2003).

Nitrogen is essential for plants since it can promote rapid growth, enhance leaf size and quality, speed up crop maturity and also help in fruit and seed development. As a main nutrient, nitrogen play role as a vital component of proteins which is nucleic acid that carry every species' genetic code. Nitrogen also is one of the components of chlorophyll that enable photosynthesis process. Recent studies prove that nitrogen-fixing bacteria can enhance growth and production of various plants significantly (Aseri *et al*., 2008).

Plants show deficiency symptoms when there are nutrients depletion occurs. For nitrogen deficiency, plants exhibit slow stunted growth and their foliage become pale green. In addition, low nitrogen uptake result in low yield and quality in leafy crops such as tobacco and vegetables (Tucker, 1999). Usually, nitrogen deficiency happens because of insufficient fertilizer application, denitrification by soil microbes, or leaching loss as a result of excessive rainfall. Sandy-textured coastal soil is a common type of soil that leaching can be take place after excessive rainfall. Besides, volatilization of nitrogen from surface during hot and weather also can cause nitrogen deficiency.

2.1.2 Phosphorus

Previous study shows that only 1.0 to 2.5% of phosphorus in the soil that can be absorbed by plants. Although phosphorus uptake by plants is less compared to nitrogen and potassium, normal plant growth cannot be achieved without it. Figure 2.2 show the phosphorus's cycle in the soil environment. Desorption, weathering of mineral phosphorus, mineralization through microbial phosphorus, crop residue and fertilizer contribute to the availability of nutrient phosphate in the soil that eventually will be uptake by plants. Plants have many potential mechanisms to increase phosphate uptake from soil including regulation of phosphate membrane transport systems, the increased growth of root hairs, enhanced mycorrhizal association, the release of phosphatases, changes in root architecture and the release of organic acids (Palomo, 2005).

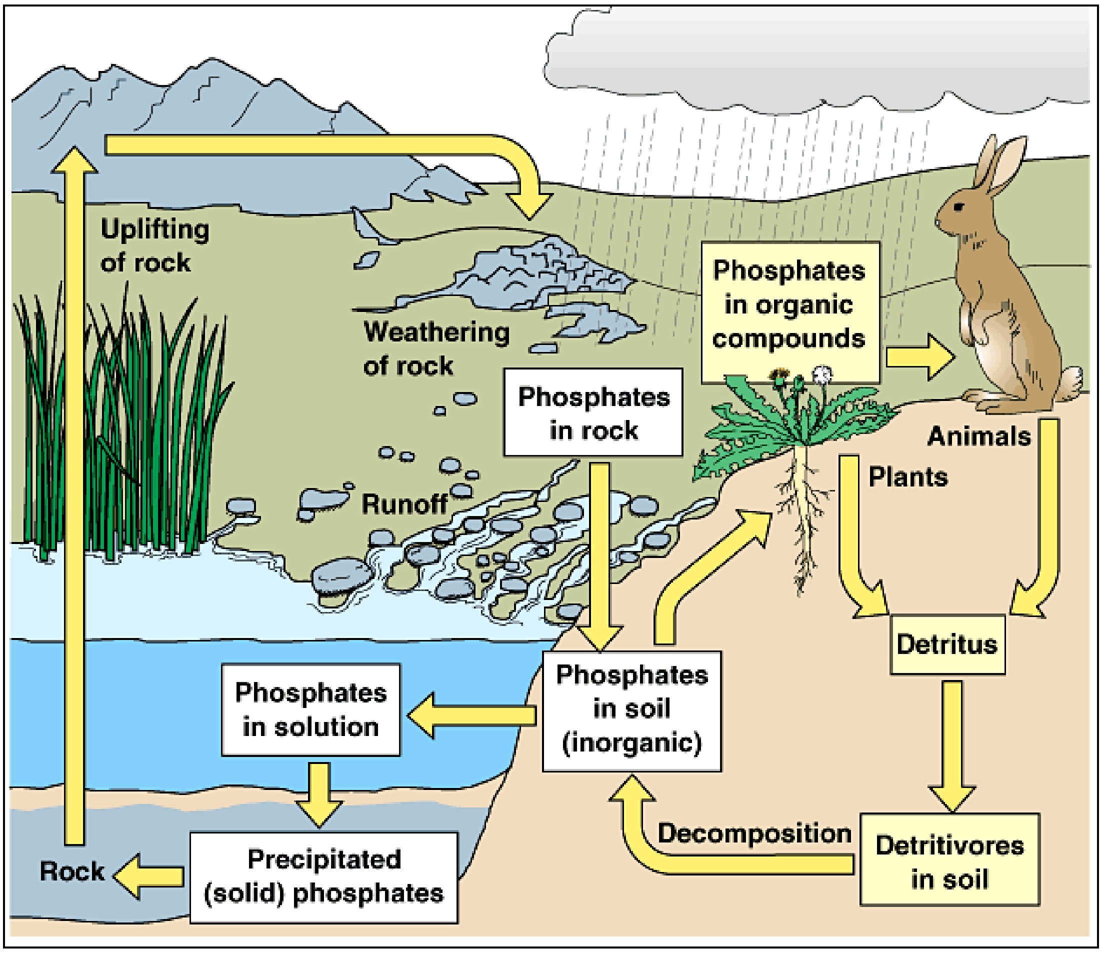

Figure 2.2: Phosphorus' Cycle (Spiro and Stigliani, 2003)

Phosphorus in form of phosphate is an essential constituent of nucleic acid, phospholipids, the coenzymes of deoxy-ribonucleic acid (DNA) and nicotinamide adenine nucleotide phosphate (NADP), and most importantly adenosine triphosphate (ATP) that supplies energy utilized throughout the plant (Tucker, 1999). Thus, phosphorus often known as "energizer" since it helps store and transfer energy. Besides, phosphorus can activates coenzymes for amino acid production used in protein synthesis and decomposes carbohydrates produced during photosynthesis. Furthermore, phosphorus play important role in many other metabolic processes which are required for normal growth for example glycolysis, respiration and fatty acid synthesis. Last but not least, phosphate not only increase seed germination and early growth, it also can stimulates blooming, hastens maturity, enhance bud seed formation and aids in seed formation.

To be available as nutrient to plants, phosphorus must be present in the soil solution as the anions of $H_2PO_4^{2-}$ or HPO_4^- . On the other hand, most other forms are low solubility which can lead to deficiency of this element. Moreover, various properties of phosphate such as weak mobility, formation of insoluble forms with many cations and fixation in organic soil compounds can also lead to deficiency (Hirsch, 2006). Therefore, previous study has tried to provide bio-fertilizer with phosphorus rock to optimize eggplant growth. However, the eggplant growth not significantly increases (Han and Lee, 2005). Phosphorus deficient will affect major energy storage and transfer functions of plants since ATP is mobile within the plants. Besides, it also disturbs root development and cause early flowering and ripening especially when the temperature is low. Then, the symptoms are characterized by undersized growth, dark green leaves with a leathery texture, and reddish purple leaf tips and margins. Usually, young plants are effected and show the symptoms when the temperature is below 60°F.

2.1.3 Potassium

Potassium is available in four forms in the soil which are K ions (K^+) in the soil solution, as an exchangeable cation, tightly held on the surfaces of clay minerals and organic matter, tightly held or fixed by weathered micaceous minerals, and present in the lattice of certain K-containing primary minerals. Based on Figure 2.3, it shows the potassium's cycle in the soil-plant-animal system.

There are several processes that contribute to the availability of potassium in the soil. Soil solution potassium is already available in the soil for plant uptake, however the concentration of potassium is affected by soil weathering, cropping history and fertilizes use. Thus, the amount present is insufficient to meet crop requirement. Then, indicator of soil potassium status such as exchangeable potassium has rapid equilibrium with the soil solution potassium and it is considered as readily available. Fixed and lattice potassium can be grouped together and make up the pool of non-exchangeable inorganic potassium in the soil.

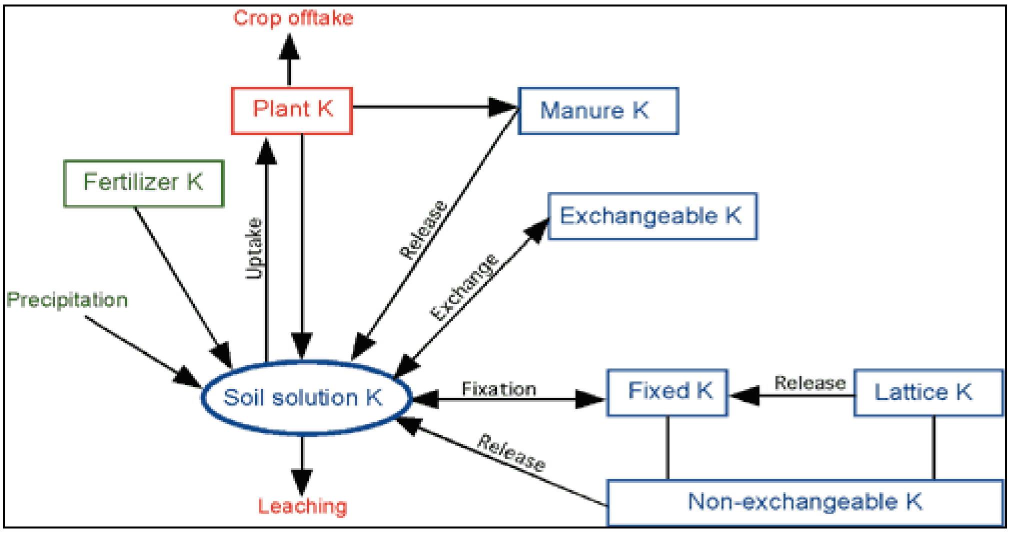

Figure 2.3: Potassium's Cycle in Soil-Plant-Animal System (Syers, 1998)

Fundamentally, K^+ is very water soluble and highly mobile and transported in the plants' xylem (Lack and Evans, 2005). Membrane transport of potassium can be mediates either by potassium channels, utilizing the membrane potential to facilitate transport of potassium down its electrochemical gradient, or by secondary transporters'. In plants, potassium act as regulator since it is constituent of 60 different enzyme systems of drought tolerance and water-use efficiency. All crops required potassium especially high carbohydrate plants such as bananas and potatoes (Hillel, 2008). In addition, current study has showed that to optimum growth, crops need more potassium than needed (Simonsson *et al*., 2007). This means that the uptake of potassium depends on the rate at which it supplied through roots rather than amounts of potassium availability in the soil. Although it is not an integral part of cell structure, potassium regulates many metabolic processes required for growth, fruit and seed development.

Potassium has many functions in plants growth such as smooth the progress of cell division and growth, increase disease resistance and drought tolerance, regulate the opening and closing of the stomata and required for osmotic regulation. Besides, potassium is essential for photosynthesis process and act as key to activate enzymes to metabolize carbohydrates for the manufacture of amino acids and proteins. Furthermore, potassium assimilates transport during plant ontogeny and one of the most important influences is improve oil content in plants.

Since the use of potassium is covered a lot of plants activities, depletion potassium uptake can cause problem for plants growth. A deficiency of this element also known as potash deficiency exhibit chlorisis (loss of green colour) along the leaf margins. Then, in severe cases, leaf will turn into yellow colour and eventually will fall off. It also affects plant growth and canopy photosynthesis process. There are several factors that lead to this problem for instance low soil potassium supplying capacity, insufficient application of mineral potassium fertilizer and bio-fertilizer, complete removal of plant straw, leaching losses and phosphorus and nitrogen deficiency (Das *et al*., 1980). Furthermore, the presence of extreme amounts of reduced substance in poorly drained soils will cause retarded root growth and reduced K uptake.

There are plenty of deficiencies symptoms associated with potassium. Some of them are older leaves change from yellow to brown, leaf tips and margins dry up, poor root oxidation power, discolourization of younger leaf, unhealthy root system that may cause reduction other nutrient uptake, and reduce cytokinin production in roots. Based on specific crops type of potassium deficiency, grain crops such as corn become small in size and have low yield and tomatoes exhibit uneven fruit ripening. Besides, cotton leaves turn reddish-brown, appear scorched, become bronze then black, and eventually fall while yield of forage crops is low and its quality is poor.

In tropical country such as Malaysia which receives rainfall every year, the potential of potassium leaching in soil is high. Thus, study of potassium uptake and investigate how to enhance this element uptake is necessary.

2.2 Biofertilizer

Biofertilizer is a type of fertilizer which use beneficial microorganisms that can be applied to seed, root or soil and function to mobilize the availability of nutrients especially NPK by their biological activity in particular, and help build up the micro-flora and enhance the soil health in general. Different microorganisms used to supply different kind of nutrients in the soil. For examples, N-fixer microorganisms such as *Azotobacter chroococcum* can supply nitrogen by fixing the nitrogen from atmosphere and convert the nitrogen into ammonium ion for plants' uptake. Besides, *Basillus megaterium* is one of the phosphorus solubilizer that apply in biofertilizer to solubilize phosphorus soil and rock in form of phosphate ion. Then, KSB for instance *Basillus mucilaginosus* is function to solubilize potassium rock and can stimulate plant growth through synthesis of growth promoting substance.

Through these microorganisms' activities, nutrients will be supply in constant and sustainable way compared to the chemical fertilizer. This is because biofertilizer can improve the physical properties of the soil and enhance water holding capacity. Microorganisms' activities are occur in the rhizosphere and via their activities can construct good and health rhizosphere by having constant and sustainable production of nutrient to be supplied into the plants' root. Moreover, microorganisms that supply by biofertilizer can prevent nutrient leaching while adding nutrients to the soil via their activities. On the other hand, chemical fertilizer also supplies nutrients such as NPK nutrients and in higher amount compared to the biofertilizer. However the nutrients supplied only can be used for instant time because easy to leaching by weathering. Recent study have proved that the used of biofertilizer by combining 25% of chemical fertilizer bring a good result for plants' growth in long term period (Kramany *et al.*, 2007).

Nowadays, biofertilizer is an alternative to chemical fertilizer to increase soil fertility and crop production in sustainable farming. Furthermore, the use of biofertilizer has gained momentum in recent years since chemical fertilizers are high cost and can cause hazardous effect (Aseri *et al.*, 2008). There are other advantages of using biofertilizer are low cost, lead to soil enrichment with nutrients, compatible with long term sustainability and eco-friendly. However, despite of these advantages, biofertilizer do not produce quick and spectacular responses. Then, the amount of nutrients provided is inadequate to meet the total needs of crops for high yields. Nevertheless, recent studies have proved that biofertilizer can increase crop yield. For example, by introducing KSB and phosphate solubilizing bacteria (PSB), primary macronutrient of nitrogen, phosphate and potassium uptake is increase on eggplant, pepper and cucumber and lead to higher yield (Han *et al*., 2006).

2.3 Rhizobacteria

The term rhizosphere is referring to a habitat for interactive community of microorganisms and a region under the influence of the root. It includes the rhizoplane (surface of the root) and the endorhizosphere (intercellular space between the root tissues inhabited by non-symbiotic bacteria). Usually, plants growth is influenced by continuous interaction between the plants root and microorganisms (Zaidi *et al*, 2003). There are a lot of microorganisms thriving in the soil, especially in the rhizosphere of plants. They are important components in the sub soil ecosystems since they contribute not only for nutrient availability but also bind soil particle into stable aggregates. This led to improvement of soil structure and reduces erosion potential.

These bacteria are known as Plant Growth Promoting Rhizobacteria (PGPR) and were first defined by were first defined by Kloepper and Schroth (1978). These bacteria and microflora in the soil are capable to solubilize or metabolize minerals, fix atmospheric nitrogen, secrete antibiotics or compounds promoting plant growth and compete or induce plant pathogen resistance. Besides, PGPR form a symbiotic relationship with many legumes through mutualism which is beneficial for both parties. PGPR develop plant growth via direct and indirect means but the specific mechanisms of action have not been well defined and characterized. Usually, by absence of pathogens or other PGPR, direct mechanisms of plant growth promotion by PGPR can be demonstrated. Then, indirect mechanisms involve the ability of

PGPR to reduce effect of pathogens on crop yield. PGPR have been reported to directly enhance plant growth by a variety of mechanisms such as fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones.

There are several types of rhizobacteria and the type is depending on the nutrients provided into the soil systems and mechanism used. Nowadays, biofertilizer are able to increase plants' nutrients uptake by introducing nitrogen fixing bacteria associated with roots (*Azospirillium*) for nitrogen uptake, iron uptake from siderophore producing bacteria (*Pseudomonas*), sulfur uptake from sulfur-oxidizing bacteria (*Thiobacillus*), phosphorus uptake from phosphate-mineral solubilizing bacteria (*Bacillus*, *Pseudomonas*) and potassium uptake from potassium solubilising bacteria, KSB (*Bacillus*). These are the several types of PGPR that usually used in the biofertilizer and introduce into the soil and their mechanism take place at the rhizosphere.

In this research study, KSB is used to study the optimum growth condition and hopefully can enhance potassium uptake of the plants when optimum condition of KSB is applied when planting. There are still no researches about KSB growth since the main nutrients are nitrogen and phosphorus. Usually, there are two types of KSB used in the biofertilizer which are *Bacillus edaphicus* and *Bacillus mucilaginosus.* For this experimental study, KSB is isolated from biofertilizer itself and had been culture in agar slant. KSB can be cultured in many type of nutrient medium such as sucrose-minimal salt medium and Luria-Bertani (LB) broth.

Fundamentally, KSB is a heterotrophic bacterium which is obtaining all their energy and cellular carbon from pre-existing organic material. Thus, they are important in the formation of humus in soil, the cycling of other minerals tied up in organic matter, and the prevention of build up of dead organic materials. Besides, KSB are aerobic bacteria which play an important role in maintaining soil structure by their contribution in the formation and stabilization of water-stable soil aggregates. In addition, this gram positive bacterium can produce substance that stimulate plant growth or inhibit root pathogens (Egamberdiveya, 2006). Moreover, KSB specifically are well known for its capability to solubilize rock potassium mineral such as micas, illite and orthoclases. This is done through the production and excretion of organics acids. (Han *et al*., 2006). Therefore, KSB are function to increase potassium availability in soils besides increase mineral contents in plants. This kind of bacteria is very useful study although potassium is not main nutrient compared to nitrogen and phosphorus, since plant will not growth steadily without potassium.