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Isolation, characterization and identification of effective microorganisms from Malaysia biofertilizer products

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Research Paper

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Nowadays, the use of biofertilizer in agriculture is an alternative approach in sustainable farming in order to sustain the balance of natural ecosystem. There are various biofertilizer products in Malaysia market that claimed to have effective and beneficial microorganisms possibly to enhance the plant growth. Therefore, the purpose of this study was to isolate, characterize and identify the effective microorganisms from biofertilizer and also to find out its functionality and present. Seven different microbes were successfully isolated from four types of biofertilizer products: sample SR1 Bunga, sample SG-1, sample SKT-1 and sample SKT-2. Results from gram staining showed that all isolates were gram positive bacteria. Three types of tests were conducted to identify their functionality. Nitrogen fixation ability test was carried out using nitrogen-free media by observing the colour changes of growth media around the bacteria colony from green to blue. Phosphate solubilization test was conducted using Pikovskaya's agar media by observing the formation of clear halo zone, while the Indole acetic acid (IAA) production was carried out by adding the tryptophan and checked the optical density of the colour changes in the broth media. These isolates were identified by 16S rDNA determination. *Bacillus* and *Staphylococcus* groups are the most dominant strain can be found in these products, These bacteria have potential to fix atmospheric nitrogen, able to produce IAA with the range of 15.13± 0.2 to 33.1± 0.2 µg/ml when supplemented with 100 µg/ml of tryptophan, and showed some P-solubilizing activity which have been claimed by manufacturers.

Key words: Biofertilizer, isolation, indole acetic acid, (IAA), nitrogen fixation, phosphate solubilization

INTRODUCTION

The intensive application of chemical fertilizers in agriculture has caused damage to the agriculture ecological system, and in long run may cause serious degradation in soil quality, water supplier, and contributing to environmental pollution. Thus, this prompted the possibility for replacing those chemical fertilizers with biofertilizer. Biofertilizers are environmental friendly fertilizers that not only prevent damages to natural sources but help, to some extent, in cleaning the nature from precipitated chemical fertilizers (FAO, 2008). The use of organic matter such as sawdust, rice bran, rice husk and shredded paper in producing biofertilizer is economical. They also act as the carrier material for nutrient and microorganisms. Biofertilizers can be considered as key components of integrated nutrient management (Mohammadi and Sohrabi, 2012). The term "Biofertilizer" or more appropriately "Microbial inoculants" can generally be defined as preparation containing live or latent cells of efficient strains of Nitrogen fixing, Phosphate solubilising or cellulolytic microorganisms used for application to seeds, soil with the objective of increasing the number of such microorganisms and

accelerate those microbial process which augment the availability of nutrients that can be easily assimilated by plants (Pandit et al., 2011).

The action modes of plant growth promoting rhizobacteria (PGPR) inbiofertilizer is by enhancing the nutrient uptake by the host plant through five areas: (1) biological nitrogen fixation, (2) increasing the availability of nutrients in the rhizosphere, (3) inducing increases in root surface area, (4) enhancing other beneficial symbioses of the host, and (5) combination of modes of actions. These PGPR used in biofertilizers including nitrogen fixing bacteria, phosphate-solubilizing microbes and mycorrhizae which are able to fix atmospheric nitrogen or solubilize phosphorus in the soil (Vessey, 2003; Subba, 1999). They are from genera as,*Alcaligenes*,*Acinetobacter*,*Arthrobacter*,*Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavo bacterium*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, and *Serratia* (Sharma et al., 2011).They can fix atmospheric nitrogen into ammonia and assimilate it into amino acid (Moat et al., 2002).The three common types of nitrogen fixing bacteria that used in biofertilizer are symbiotic nitrogen fixing bacteria, nitrogen fixing associated bacteria and free-living nitrogen fixing bacteria (Alexander, 1961). After nitrogen fixation, phosphate solubilisation is a very important plant growth promoting activity. Several soil bacteria particularly belonging to genera *Bacillus* and *Pseudomonas*, possess the ability to change insoluble forms into soluble form by secreting organic acids as formic acid, acidic, propionic, lactic, glycolic, fumaric and succinic acid (Vazquez et al., 2000). According to Sharma et al. (2011) phosphorus solubilizing bacteria play an important role in phosphorus nutrition by enhancing its availability to plants through the release from inorganic and organic soil phosphorous pools, by solubilisation and mineralization, andincrease the nutrient uptake of the plant by producing various organic acids, siderphores, mineral acids, protons, humic substances, carbon dioxide and hydrogen sulfide (Ivanova et al., 2006).

Recently, there are many biofertilizer products in the market which claimed to have beneficial microbes such as *Bacillus*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Staphylococcus*and certain *actinomycetes*; so that these biofertilizers can help in plant nutrient uptake to enhance the growth (Subba, 1999).However, in certain condition, these biofertilizers did not show any improvement on the crop yields. Thus, laboratory assessment of these products need to be carried out to identify and quantify the microbes present in the products.

MATERIALS AND METHODS

Isolation of microorganism from biofertilizer products (15:15:15)

The microbial contents of biofertilizer were quantified and isolated from the local biofertilizer products (Sample 1: SR1 Bunga; Sample 2: SG-1; Sample 3: SKT-1, Sample 4: SKT-2) by using different growth media: MRS agar, potato dextrose agar and nutrient agar (Merck, Germany). A serial dilution of isolated bacteria were inoculated into different agar and incubated at 30-33°C for 24 h according to the standard protocol (John, 1994).

Gram staining

The samples were heated fixed on the slide through the Bunsen burner for three times. Crystal violet was added onto the slide and incubated for one minute. The slideswere then rinsed gently with stream of water for 5 seconds to remove unbound crystal violet. Gram's iodine was added later with the purpose of fixing and gently removed by stream water.

The alcohol was added to remove the crystal violet. Finally Safranin was added for 1 min and gently removed by stream water. The slides were observed under microscope (John, 1994).

Determination of nitrogen-fixing bacteria by the spread plate method

Nitrogen fixation ability of isolated samples were tested by growing them on nitrogen-free solid malate medium (Nfb medium) [(g/L): DL-malic acid, 5; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; KOH, 4; NaCl, 0.1; $CaCl_2$, 0.02; Trace element solution, 2; 5 % Bromothymol blue, 2; Fe-EDTA, 4; vitamin solution, 1] (Döbereiner and Day, 1976). 10 ul of isolates was pipetted from nutrient broth onto the N-free medium and incubated for 24 h at 30-33 °C. The ability of isolates to fix nitrogen was observed by colour changes of medium.

Phosphate solubilization

The isolated bacteria were grown on Pikovskaya agar medium [(g/L): Glucose, 10; $Ca_3(PO_4)_2$, 5; $(NH_4)_2SO_4$, 0.5; NaCl, 0.2; $MgSO_4.7H_20$, 0.1; KCl, 0.2; yeast extract, 0.5; $MnSO_4.H_2O$, 0.002; $FeSO_4.7H_2O$, 0.002 and agar, 15. 5 µL (Pikovskaya, 1948)]. The isolates in nutrient broth was pipetted onto sterile disk and put onto the agar medium, then incubated for 24 h at 30-33ºC. The diameter of the clear zone around the colony was observed and recorded.

Indole acetic acid (IAA) production

The NFb broth containing 0.2 g/litre yeast extract, 1 g/litre NH_4SO_4 and 100 mg/litre of tryptophan, was inoculated with 1% (v/v) cell suspension and incubated in the dark condition with agitation at 150 rpm at 30ºC for 48 h. The

IAA production of each isolate was measured according to the Salkowski colorimetric technique as described by Glickmann and Dessaux (1995).

16S r DNA gene amplification and sequencing

A loopful of bacterial cell was picked up and suspended in TE buffer [100 mM Tris (pH 8.0), 30 mM EDTA (pH 8.0), 360 µl]. Lsozyme (50 mg/ml, 20 µl) was added into the tube and incubated at 37°C for 30 min. SDS [10% (w/v), 40 µl] was added, and the tube was inverted for 5-6 times, then incubated at 55°C for 10 min. Phenol: chloroform: isoamyalcohol [25:24:1, 400 µl] was added and mixed well. The mixture was spun at 13,000 rpm rpm for 15 min. The upper solution was transferred to new microtube (Sambrook and Russell, 2001). The same procedure was repeated if the solution was not clear. 3 M Sodium acetate (1/10 volume) and 2 volume of cold ethanol was then added. DNA was then pooled by glass rod and dried for 5-10 min until DNA became clear.

Finally, the DNA was dissolved in 100-200 µl of sterilized distilled water and stored at -20 °C. Polymerase Chain Reaction (PCR) of the 16S rDNA for the bacterial isolates was conducted and the PCR reaction was carried out as followed: sterile distilled water (59.5 µl); 10X PCR buffer (Promega, USA, 10.0 µl); 25 mM $MgCl_2$ (Promega, USA, 8.0 µl); 2 mMdNTP mix (Promega, USA, 10.0 µl); universal primers (Forward: 5' GAG TTT GAT CCT GC TCA G 3'; Reverse: 5' GTT ACC TTG TTA CGA CTT 3'] Invitrogen, USA, 10 pmol/µl, 4.0 µl) ; Taq Polymerase (5U/µl; 0.5 µl) and genomic DNA as template (4 µl). The PCR tubes was then put into therma cycler and preheat at 94°C for 3 min, followed by 25 cycles of denaturing at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and final elongation at 72°C for 3 min. The reactions were kept at 4 ºC until loaded onto the gel. The PCR product was purified, sequenced andcompared with the 16S rDNA sequence of bacteria from the NCBI Gene Bank nucleotide sequence database (http://www.ncbi.nih.gov).

Statistical analysis

All experiments were carried out in triplicate and the measurement were subjected to analysis variance (ANOVA) and significance at the 5% level was tested by Least Significant Difference (LSD) using SAS package, version 8.2 (SAS, 2001).

RESULTS AND DISCUSSION

Seven strains of bacteria were isolated from four samples of biofertilizer products. Three strains were isolated from sample SRI Bunga, two strains from sample SG -1, one strain from sample SKT-1 and sample SKT-2. All the bacteria were characterized and differentiated based on their different characteristic and physical morphology in size, surface, texture, color, elevation and margin. The morphology of each identified bacteriawere showed in (Table 1). Based on these preliminary data, *Bacillus* and *Staphylococcus* groups are the most dominant strains which can be found in all types of biofertilizer products.71% ofbacteria gave positive response towards the nitrogen fixation study, similar finding from Tan et al (2009) which indicated by colour changes of the medium from pale green to blue (Figure 1). The diameter of colour changes ranges from 0.73 to 3.13 cm.

This is due to the increase in pH by formation of ammonia and nitrates from the atmospheric N_2 fixation (Döbereiner and Day, 1976). As known that 80% of the atmosphere is made up of nitrogen gas that is unusable for most living organisms. Biological nitrogen fixation (BNF) is the changes of nitrogen gas to the form of useful NH_3 that can be taken up by plant. As nitrogen fertilizer is one of the major nutrients needed by plant, biofertilizerenhance the productivity and sustainability of soil as they are low cost, eco-friendly and renewable source of plant nutrients. Thereby, they play an important role in sustainable agricultural system. In recent years there has been an upsurge into the research related to biofertilizers since they act as natural stimulators of plant growth and development.

Consequently, there is considerable interest in the possible use of inoculants of effective microbes for the development of biofertilizer. This mainly involves the selection and multiplication of plant-beneficial microorganisms such as bacteria, algae and fungi, either alone or in combination (Vazquez et al., 2000). However, the formulation of biofertilizer also play important role. More study needs to be carried out for stabilization and protection of microbial cell during storage, transport and at the target plants (Brahmaprakash and Sahu, 2012).For those bacteria which did not form the colour changes of the medium still can be found in the biofertilizer products because they may be contributed for the production of phytohormones which important for plant growth and contribute for antimicrobial activities.

From this study, the microbial contents of all biofertilizer products dominated by N_2-fixing associated bacteria which belong to Bacillus group. There are no genera of *Rhozobium*, *Bradyrhizobium* or *actinomycetes* appeared in the products as claimed by manufacturers. This *Bacillus* group not only potential to fix nitrogen, they also have capable to produce plant growth hormones, solubilize phosphate and producing degrading compound such as pectin (Dommergues, 1992).

These bacteria also produced different amount of Indole acetic acid (IAA) in the broth culture with the range of 15.13 ± 0.2 to 33.1 ± 0.2 µg/ml when supplemented with 100 µg/ml of tryptophan (Table 1). As known that in the natural ecosystem, soil microbes always produce a variety of substances which directly or indirectly affect

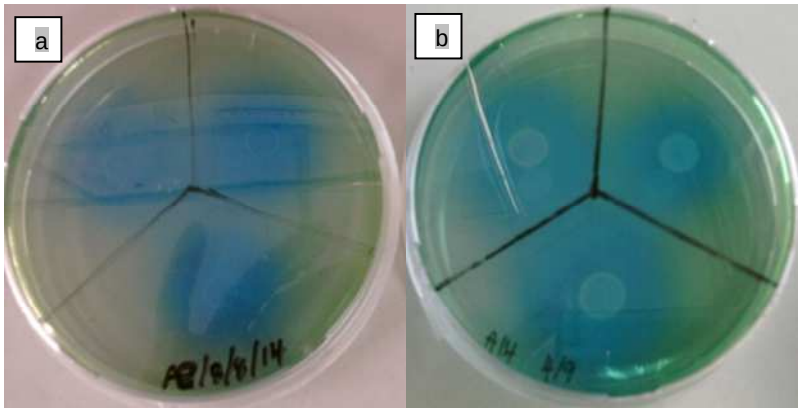

Figure 1. The colour changes of the nitrogen-free medium around the colony were observed from pale green to blue. (a) *Bacillus tequilensis,* (b)*Bacillus methylotrophicus.*

Table 1. Biofertilizer products, isolate number, identification name, description and functionality of the isolated bacteria strains. Bacteria strains were assayed for Nitrogen Fixing Bacteria (NFB), Phosphate Solubilizing Bacteria (PSB) and Indole acetic acid producer (IAA), after 24 hours of incubation at 30-33 °C

Products	Isolates No	ID	Functionality ($LSD_{0.05}$)*	Description
SR1 Bunga	A1	*Bacillus cereus*	IAA^{dc}, NF^{f}	Irregular form, diameter size is around 4 to 5 mm, has glistening surface, moist texture, cloudy and white in colour, raised elevation and lobate margin.
SR1 Bunga	A4	*Staphylococcus sp.*	IAA^{a}	Circular form. Its diameter size is 3 to 5 mm, has glistening surface, moist texture, yellowish green, cloudy, umbonate elevation and entire margin.
SR1 Bunga	A5	*Staphylococcus saprophyticus*	NF^{d},PSB^{a}, IAA^{dc}	Circular form. Its diameter size is 2 to 3 mm, has glistening surface, moist texture, white in colour, cloudy, umbonate elevation and lobate margin.
SG-1	A10	*Bacillus sp.*	Nil	Circular form. Its diameter size is 2 mm. It has dull surface, moist texture, white-pale and cloudy, flat elevation and entire margin.
SG-1	A11	*Bacillus tequilensis*	NF^{c}	Circular form. Its diameter size is 4 mm, has rough surface, moist texture, cloudy, flat elevation and entire margin.
SKT-1	A14	*Bacillus methylotrophicus,*	NF^{a}, IAA^{d}	Regular form, glistening surface, viscous and translucent
SKT-2	A16	*Bacillus amyloliquefaciens*	NF^{d}, IAA^{ab}	Irregular form. It has glistening surface, viscous texture, translucent, raised elevation and undulate margin

* Analysis variance (ANOVA) and 5% level was tested by Least Significant Difference (LSD).

during the growth. In normal condition, these hormones(secondary metabolites) will be secreted when the bacteria reach the stationary stage where the utilization be secreted when the bacteria reach the stationary stage where the utilization of carbon is at the saturated level (Subba, 1999).

The P-solibilizing activity of one isolates from biofertilizer products was tested in agar medium (Figure 2). There was only 14% of bacteria showed positive response towards phosphate solubilization ability test, which indicated by the formation of the clear halo zone around the colony. The rest did not show any significant results for the test (Table 1). This study indicates that there are some P-solubilizing bacteria occurred in these biofertilizer products as claimed by manufacturers. There are numbers of soil bacteria posse mineral phosphate solubilizing activity naturally (Mikanova and Kubat, 1994). However, the activity always been affected by the presence of soluble phosphates in the rhizosphere soil (Mikanova and Novakova, 2002). Thus, the regulation of P-solubilizing activity was completely inhibited by appearance of soluble phosphate in the soil. The additional large proportion of phosphorus becomes insoluble, and thus unavailable of nutrient uptake by the plant (Singh and Kapoor, 1994; Peixet al., 2003). Therefore, the additional application of biofertilizer into

Figure 2. The clear halo zone around the colony were observed for phosphate solubilisation activity of the *Staphylococcus saprophyticus.*

the soil can help mineral phosphorus fertilization and nutrient uptake by the plants.

Conclusion

This study indicated that *Bacillus* and *Staphylococcus* are the most dominant strains can be found in four types of local biofertilizer products. They have potential to fix atmospheric nitrogen, able to produce IAA, and showed some P-solubilizing activity which have been claimed by manufacturers.

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