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Isolation of beneficial microbes from biofertilizer products

(Pengasingan mikrob berfaedah daripada produk baja biologi)

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Abstract

Since the uses of chemical fertilizers have threatened human health and food safety, many people have approached the use of biofertilizer as an alternative input for plant growth in order to maintain soil and water quality, and sustain the natural ecosystem. There are various biofertilizer products in the market which claimed to have beneficial microbes and effective for plant growth. Thus, this study was conducted to identify and assess the microbial contents of various biofertilizer products available in the market. A total of 13 isolates of bacteria were identified from these biofertilizer products which were imported from Thailand, China and Australia. *Bacillus* group was the most dominant strain found in these products, while the other bacteria belong to genera *Azospirillum*, *Corynebacterium*, *Pseudomonas* and *Proteus mirabilis*. These bacteria have potential to fix atmospheric nitrogen, able to produce IAA with the range of 3.75–12.2 mg/litre when supplemented with 100 mg/litre of tryptophan, and showed some P-solubilizing activity (5.7–9.0 mg/litre).

Introduction

Soil infertility is one of the most important factors contributing to lower crop yields world-wide especially in developing countries. Intensive application of chemical fertilizers in agriculture has caused damage to the ecological state of agricultural system, and in long run may cause serious environmental pollution, degradation in soil quality and water supply. Thus, this prompted the possibility of replacing those chemical fertilizers with biofertilizer.

Biofertilizer contains living microorganisms and promotes growth by increasing the availability of primary nutrient (nitrogen and phosphorus) to the host plant (Vessey 2003). Biofertilizer also provides nutrients required by the plants and helps to increase the soil quality with natural microorganism (Vessey 2003). It is environmental friendly and has the ability to convert nutritionally important elements from unavailable forms to available forms through biological process. It also acts as a soil conditioner, which improves air and water relationship in soil and makes soil less prone to compaction and erosion.

Some of the beneficial microbes used in biofertilizers are N_2-fixing bacteria, phosphate-solubilizing microbes and mycorrhizae which are able to fix atmospheric nitrogen or solubilize phosphorus in the soil (Subba 1999). The three types of N_2-fixing bacteria used in

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biofertilizer are symbiotic N_2-fixing bacteria, N_2-fixing associated bacteria and free-living N_2-fixing bacteria.

Some examples of symbiotic N_2-fixing bacteria are *Rhizobium* and *Bradyrhizobium*, which infect leguminous plant such as bean, pea, alfalfa, soybean and cowpea (Alexander 1961). Meanwhile, *Herbaspirillum*, *Azotospirillum, Acetobacter diazotrophicus* and *Pseudomonas* are examples of N_2-fixing associated bacteria in sugar cane, sorghum, rice and maize (Lee et al. 1996; Rasul et al. 1996). The growth response of free-living N_2-fixing bacteria was due to the bacterial synthesis of secondary growth-promoting compounds. Obligate aerobic bacteria belong to the genera *Azotobacter, Beijerinckia*, *Derxia*, *Achromobacter*, *Mycobacterium*, *Arthrobacter* and *Bacillus*, while anaerobic nitrogen-fixing bacteria are represented by genera *Clostridium*, *Chlorobium*, *Chromatium*, *Rhodomicrobium*, *Rhodopseudomonas*, *Rhodospirillum*, *Desulfovibrio* and *Methanobacterium* (FAO 1982).

Besides nitrogen, certain bacteria and fungi also have the ability to solubilize organic phosphorus in the soil by producing acid phosphatase, formic, acetic, propionic, lactic, glycolic, fumaric and succinic acids to accelerate the nutrient uptake from the plants. Those bacteria belong to the genera *Bacillus*, *Pseudomonas*, *Aspergillus* and *Penicilium* (Gaur 1990). At the same time, certain bacteria such as *Bacillus*, *Pseudomonas* and *Azospirillum* are reported to produce plant growth hormones such as indole acetic acid (IAA), gibberellin, cytokinins and ethylene (Rasul et al. 1996). These hormones contribute to overcome the dormancy and dwarfism in plants; induce flowering in some photoperiodically sensitive plants and other low temperature dependent plants; alter the sex of flowers and contribute to fruit setting; and stimulate stem growth and at the same time suppress the growth of lateral branches.

Recently, there are various biofertilizer products in the market which claimed to have beneficial microbes such as *Bacillus*, *Azotobacter*, *Azospirillum*, *Pseudomonas* and certain actinomycetes. However, the assessment of these biofertilizer products need to be done to identify and quantify the microbes present in the products. Thus, this study was carried out to isolate and characterize these bioproducts.

Materials and methods

Isolation of microorganism from biofertilizer products

The microbial contents in biofertilizer were quantified and isolated from the imported biofertilizer products (Thailand, China and Australia) by using different growth media: MRS agar, NFb agar, LGI agar, PDA and actinomycetes agar. A serial dilution of isolated bacteria was inoculated into tubes with 5 ml of NFb and LGI N-free semi-solid medium with bromothymol blue (Döbereiner and Day 1976) and incubated at 37 °C for 3–5 days. The white veil-like pellicle below the surface of the semi-solid medium was purified. The pure cultures were maintained on nutrient agar slant at 4 °C. The quantification of cfu/ml was performed after incubation on nutrient agar for 24–48 h at 37 °C.

For metabolic profiles, the isolates were obtained using the Microlog system (Version 4.2, 1993, Biolog Inc, USA.). Biolog Gram Positive (GP) and Gram Negative (GN) were used to identify Gram positive and Gram negative bacteria. A standard inoculum was determined with a turbiditimeter and a suspension was prepared by removing the cells from a plate (BUG agar) with a sterile swap onto inoculating fluid [Sodium Chloride, 0.4 % (w/v); Pluronic 7–68 0.03 % (w/v); Gellan Gum 0.02 % (w/v)]. The cell density was adjusted to 20% ± 2 transmittance for Gram positive rod-shaped non-spore forming bacteria. About 150 µl of the suspension was inoculated into the microplate containing 95 carbon sources with multi-channel pipette. The Gram positive rod-shaped non-spore forming bacteria were incubated

16–24 h in the anaerobic container at 35 °C. The biochemical fingerprint was automatically read with Micro Station Reader using Micro Log3 Software version 4.2. The comparison was made against the database containing identification patterns for Gram positive and Gram negative bacteria species.

Propagation of beneficial microbes

The isolated microbe was propagated using enriching non-selective medium (CPMA). The strains of each group were incubated separately for 7 days at 25 °C and then in mixture for 72 h at 37 °C under agitation at 75 rpm. The bacterial isolates were then subjected to a series Gram staining and colony count.

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 30 °C for 48 h. The IAA production of each isolate was measured according to the Salkowski colorimetric technique as described by Glickmann and Dessaux (1995).

Phosphate solubilizing by PGPRs

The liquid medium (10 g/litre glucose, 1 g/litre asparagin, 0.2 g/litre K_2SO_4, 0.4 g/litre $MgSO_4.7H_2O$ and 0.2 g/litre autolysate yeast) and 5 g/litre of Ca_3PO_4 was inoculated with 1% (v/v) cell suspension and incubated with agitation at 150 rpm at 30 °C for seven days. Analyses were performed in three replicates and the average values are presented in the table. The medium was filtered after incubation and the remaining tricalcium phosphate was separated by filtration. Medium was poured onto the filter, rinsed with hot distilled water to remove slime and soluble phosphate. Filter papers were dried for 15 min at 105 °C and then hydrolysed with $2NH_2SO_4$ for 18 h. The solution was filtered again and 2 ml of the filtrate was topped up to 100 ml with distilled water. The phosphorus was determined colorimetrically by method described by John (1970).

Results and discussion

A total of 13 strains of bacteria were isolated from commercial biofertilizer products from Thailand, China and Australia. The genus and species of each isolate were identified using Biolog MicroPlate System as showed in *Table 1*. Based on these preliminary data, *Bacillus* group was the most dominant strain which can be found in all types of biofertilizer products. However, the colony count of these bacteria isolates was around 10^2–10^3 cfu/ml as compared to the indigenous rhizobacteria isolated (10^8–10^9 cfu/ml) from rice field (Tan 2002). The variation of colony count may be due to their viability lost during the manufacturing or storage period, or they might also not competitive enough compared to other bacteria.

All species of bacteria showed the white veil-like pellicle below the surface of the semi-solid medium, except *Gordonia sputi, Proteus mirabilis, Rhodococcus globerulus* and *Sanguibacter keddieii* (*Table 1)*. The formation of this white veil-like pellicle in nitrogen free semi-solid medium indicated that these bacteria have the ability to fix atmospheric nitrogen (Döbereiner and Day 1976). Those that did not form the pellicle but still could be found in the biofertilizer products could be due to the production of phytohormone which was important for plant growth and contributed to the antimicrobial activities.

From this study, the microbial contents of all biofertilizer products were dominated by N_2-fixing associated bacteria in the *Bacillus* group (*Table 2*). There are no genera of *Rhizobium*, *Bradyrhizobium* or actinomycetes appeared in the products as claimed by manufacturers. This *Bacillus* group not only has potential to fix nitrogen, but is also capable to produce plant growth hormones, solubilize phosphate and produce

Table 1. Biolog MicroPlate reading of isolated bacteria from biofertilizer products, and the formation of white veil-like pellicle, indole acetic acid (IAA) and P-solubilizing activity by isolated bacteria

Isolate no.	Strain ID (country)	Cfu/ml	Gram stain	Probability index (%)	Similarity index (%)	White veil-like pellicle formation	IAA production (mg/litre)	Water soluble-P (mg/litre)
B7	*Azospirillum* sp. (AC)	2.1×10^4	Negative	98	0.72	+	12.0 ± 2	0.11 ± 0.1
B8	*Bacillus cereus* (AC)	1.9×10^3	Positive	99	0.73	+	8.5 ± 0.2	5.7 ± 0.1
B9	*Bacillus* sp. (ACT)	1.7×10^3	Positive	80	0.45	+	8.0 ± 0.2	6.6 ± 0.1
B4	*Bacillus oleronius* (T)	2.4×10^3	Positive	100	0.673	+	7.0 ± 2	5.8 ± 0.1
B6	*Brevibacillus* sp. (T)	1.42×10^3	Positive	86	0.47	+	4.0 ± 1	0.11 ± 0.2
B10	*Corynebacterium* sp. (C)	1.22×10^3	Positive	99	0.72	+	3.75 ± 0.2	0.10 ± 0.1
B2	*Gordonia sputi* (T)	1.3×10^3	Positive	100	0.67	–	0.22 ± 0.1	1.3 ± 0.1
B5	*Paenibacillus chibensis* (T)	1.22×10^2	Positive	100	0.673	+	12.2 ± 0.1	6.5 ± 0.1
B11	*Proteus mirabilis* (C)	1.9×10^2	Negative	100	0.73	–	10.0 ± 1	0.12 ± 0.1
B1	*Rhodococcus globerulus* (T)	2.1×10^3	Positive	90	0.59	–	0.5 ± 0.1	0.11 ± 0.2
B3	*Sanguibacter keddieii* (T)	1.2×10^3	Positive	90	0.59	–	0.45 ± 0.1	0.5 ± 0.1
B12	*Spirillum* sp. (A)	2.1×10^4	Negative	90	0.65	+	6.5 ± 0.1	0.11 ± 0.1
B13	*Pseudomonas* sp. (AC)	2.5×10^4	Negative	90	0.67	+	11.5 ± 0.2	9.0 ± 0.2

Imported biofertilizer products: A = Australia; C = China; T = Thailand

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 3.75 ± 0.2 to 12.2 ± 0.1 mg/litre when supplemented with 100 mg/litre of tryptophan. In natural ecosystem, soil microbes produce a variety of substances which directly or indirectly affect plant growth, and many species of bacteria are known to produce IAA in small amount, especially when the growth medium is supplemented with tryptophan as a precursor (Ahmad et al. 2005). The function of IAA is known to be involved in the root development, hence improves the rate of mineral and water uptake by the plant.

The production of plant growth hormones by bacteria depends on the utilization of carbon source during the growth. In normal condition, these hormones (secondary metabolites) will 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 13 isolates from biofertilizer products was tested in liquid culture (*Table 2*). The *Bacillus* and *Pseudomonas* groups showed a high P-solubilizing activity with the range of 5.7 ± 0.1 to 9.0 ± 0.2 mg/litre. The other groups did not show any significant results. This study indicated that there were some P-solubilizing bacteria occurred in these biofertilizer products as claimed by manufacturers even though dominated by only two groups of bacteria.

A number of soil bacteria posses mineral phosphate solubilizing activity (Mikanova and Kubat 1994), but the activity is normally affected by the presence of soluble phosphates in the medium (Mikanova and Novakova 2002). The regulation of P-solubilizing activity was completely inhibited by the appearance of soluble phosphate in the medium.

Microbial solubilization of hardly soluble phosphates in the soil is an important process in natural ecosystem

Table 2. Microbial contents of biofertilizer products from Thailand, China and Australia based on different categories

	Thailand	China	Australia
N_2-fixing bacteria	*Bacillus* sp. *Bacillus oleronius* *Brevibacillus* sp. *Paenibacillus chibensis*	*Azospirillum* sp. *Bacillus cereus* *Bacillus* sp. *Corynebacterium* sp. *Pseudomonas* sp.	*Azospirillum* sp. *Bacillus cereus* *Bacillus* sp. *Pseudomonas* sp. *Spirillum* sp.
P-solubilizing bacteria	*Bacillus* sp. *Bacillus oleronius* *Paenibacillus chibensis*	*Bacillus cereus* *Bacillus* sp. *Pseudomonas* sp.	*Bacillus cereus* *Bacillus* sp. *Pseudomonas* sp.
PGPR	*Bacillus* sp. *Bacillus oleronius* *Brevibacillus* sp. *Paenibacillus chibensis*	*Pseudomonas* sp. *Proteus mirabilis* *Azospirillum* sp. *Bacillus cereus* *Bacillus* sp. *Corynebacterium* sp.	*Azospirillum* sp. *Bacillus cereus* *Bacillus* sp. *Spirillum* sp. *Pseudomonas* sp.
Actinomycetes	Nil	Nil	Nil
Others	*Gordonia sputi* *Rhodococcus globerulus* *Sanguibacter keddieii*	Nil	Nil

and in agricultural soils. In the normal practice, the additional of large proportion of phosphorus becomes insoluble, and thus unavailable nutrient uptake by the plant (Singh and Kapoor 1994; Peix et al. 2003). Therefore, the application of biofertilizer into the soil can help phosphorus fertilization and nutrient uptake by the plants.

Even though *R. globerulus*, *G. sputi* and *S. keddieii* did not produce any hormones or fix atmospheric nitrogen, some literatures reported that they have potential in degrading phenol compound and polychlorinated biphenylis (PCBs) in the soil (Maeda et al. 1995; Przybulewska et al. 2006). Besides, *S. keddieii* is also able to produce chitinase (antifungal activity) which can be effectively utilized in biological pest control of fungi infection (Edwards and Seddon 2001).

Conclusion

Bacillus group was the most dominant strain found in the three types of biofertilizer products. The other bacteria were *Azospirillum*, *Corynebacterium*, *Pseudomonas* and *Proteus mirabilis*. These bacteria have the potential to fix atmospheric nitrogen, able to produce IAA with the supplemented tryptophan, and showed some P-solubilizing activity as claimed by the manufacturers. There were no actinomycetes or cellulolytic bacteria appeared in these samples. The other bacteria (*Rhodococcus globerulus*, *Gordonia sputi* and *Sanguibacter keddieii*), which did not contribute to the biofertilizer component, also present in the samples. Subsequent study is needed to determine their potential as antimicrobial agent.

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Abstrak

Disebabkan penggunaan baja kimia telah menimbulkan ancaman kepada kesihatan manusia dan keselamatan makanan, ramai pengguna telah menggunakan baja biologi sebagai pengganti baja kimia untuk mengekalkan kualiti tanah dan air, serta memelihara ekosistem persekitaran. Pelbagai jenis baja biologi yang terdapat di pasaran yang didakwa mengandungi mikrob pemfaedah dan berkesan untuk pertumbuhan pokok. Dengan itu, kajian ini dijalan untuk mengenal pasti kandungan mikrob yang ada di dalam pelbagai baja biologi yang terdapat di pasaran. Sejumlah 13 jenis bakteria telah diasingkan daripada baja biologi yang diimport dari Thailand, China dan Australia. *Bacillus* merupakan kumpulan paling dominan yang terdapat di dalam semua jenis baja biologi manakala kumpulan lain terdiri daripada *Azospirillum*, *Corynebacterium*, *Pseudomonas* and *Proteus mirabilis*. Bakteria tersebut berpotensi mengikat nitrogen, menghasilkan IAA (3.75–12.2 mg/liter) di dalam medium yang dibekalkan dengan 100 mg/liter triptofan dan menguraikan fosforus pada kadar 5.7–9.0 mg/liter.

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