Oil palm and banana root colonization potential of locally isolated nitrogen-fixing and phosphate-solubilizing bacteria

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Abstract. Oil palm and banana are the biggest commodity crop and the second largest fruit crop planted in Malaysia. Both oil palm and banana are highly nutrient-demanding crops that requires a large amount of fertilizer input. This presents an opportunity to find alternative source of nutrient that is much cheaper than the imported inorganic fertilizer. Currently, the most feasible alternative to the inorganic fertilizer is the recycling of the organic-rich oil palm empty fruit bunch (EFB) to produce EFB compost as well as the incorporation of nitrogen-fixing bacteria (NFB) and phosphate-solubilising bacteria (PSB) to the EFB compost to increase the supply of nitrogen and phosphorous to the plant at different stages of growth. Hence, the objective of this study was to isolate, screen and identify indigenous bacterium, from the root surroundings of oil palm and banana plant, with highest nitrogen-fixing and phosphate-solubilizing properties. Three NFB and PSB strains (*Enterobacter cloaceae KU886016, Burkholderia cepacia KU925862, Serratia marcescens KU925861*), were successfully isolated and formulated as biofertilizer for evaluation on oil palm and banana seedlings. *Enterobacter cloaceae KU886016* showed higher root colonization ability compared to *Burkholderia cepacia KU925862* and *Serratia marcescens KU925861*, as shown from the FESEM analysis. This finding is important as a direct indication on the suitability of using these bacteria in field application as biofertilizer. Long-term expectation is for this finding to be able to assist in reducing the dependency on imported inorganic fertilizers, reducing operational cost as well as promoting sustainable soil health.

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1. Introduction

Banana and oil palm are two nutrient-demanding crops that require a large amount of fertilizer input during both vegetative and reproductive stage. However, current high price of imported fertilizer in the world market (up to 87.1% increment from RM 739/ tonne in 2005 to RM 1383/ tonne in 2013) has resulted in the drastic increased of the production cost in oil palm and banana plantation. Therefore, an alternative source of organic-based fertilizer is needed for oil palm and banana plants to reduce the impact of high fertilizer price to ensure the competitiveness of oil palm and banana industry in world market. The oil palm empty fruit bunch (EFB) compost which consisted of high nutrient content of K, N and P has great potential to be utilized as fertilizer for oil palm and banana. This is expected to substantially reduce the use of the relatively more expensive imported inorganic chemicals as source for fertilizer. Apart from this, the application of N-fixing and P-solubilizing bacteria into the EFB compost can also further reduce the dependency on inorganic N and P fertilizers. Therefore, it is the objective of this study to isolate and identify indigenous N-fixing and P-solubilizing bacteria from the roots of mature oil palm and banana plants. Its potential for application in biofertilizer was evaluated through its ability to colonize root systems of oil palm and banana plants.

2. Materials and Methods

2.1. Banana and Oil Palm Root Sampling

The banana and oil palm root sampling sites were located in Felda Agricultural Services Sdn. Bhd. (FASSB), Sg. Tengi Selatan Research Station, Selangor and FASSB, Jengka 26 Research Station, Pahang. The root sampling plots consisted of both high fertilizer and agro-chemical input plot (bacteria isolated considered as highly adapted and able to survive under extreme condition) and from less agricultural input plot (bacteria isolated considered as highly active under less disturbed soil). The roots were sampled from healthy and good yielding banana and oil palm trees for both sampling plots. The oil palm roots were sampled from two mature oil palm farms (26 years after planting) while the banana roots were sampled from six sampling sites that were planted with three types of different banana cultivar namely *Pisang Berangan*, *Pisang Nipah* and *Pisang Cavendish*. The banana roots and oil palm roots that consisted of primary, secondary and tertiary roots were collected from the soil surface to the depth of 15 cm into the soil. Samples were taken from zone surrounding the trunk collar to the radius of 50 cm from the trunk (for banana) and 100 cm (for oil palm) using alcohol-sterilized forceps and scissors. This was carried out to avoid any influence from root samples emanating from nearby banana or oil palm trees. Five sampling points were identified from each sampling site where about 12 to 15 pieces of healthy roots (free from any pest and disease infection) were sampled from every individual tree that represents one sampling point. The collected roots samples were cut into smaller pieces about 6-8 cm in length and transferred into pre-sterilized plastic bags and properly sealed. All collected roots samples (30 for banana and 10 for oil palm) were stored at 4^{o}C prior to further use. The banana and oil palm root samples were washed under running tap water to remove the soil particles adhering on the root surface. The samples were immersed into 10% (v/v) NaClO solution for two minutes for surface sterilization followed by immersion into increasing concentrations of ethanol (50%, 70%, 90%, 100%) for 30 seconds each. Then, the samples were rinsed twice with sterilized distilled water for 1 minute each to remove residual chemicals [1]. The treated root samples were then transferred into pre-sterilized bottles and stored at 4^{o}C prior to bacterial isolation.

2.2. Isolation of Nitrogen Fixing and Phosphate Solubilizing Bacteria

Selective isolation of the indigenous nitrogen-fixing bacteria (NFB) from the oil palm and banana root samples were carried out using the Burk's Nitrogen Free (BNF) agar medium [2] while the phosphate-solubilizing bacteria (PSB) were isolated using the Pikovskaya (PKV) agar medium [3]. Both mediums were autoclaved at 121^{o}C for 15 minutes (HA240m, Hirayama) and let to cool to 40-50^{o}C prior to further use. To inoculate the root samples on the BNF and PKV medium agar, about 10-12 surface sterilized root samples of banana and oil palm that were kept in the bottle were transferred and dried on a sterilized filter paper. The root samples were cut into small sub sections about 0.5-0.8 cm of length and then cut longitudinally to splice open the root section followed by transferring onto the BNF and PKV agar mediums [1]. Three root sections were transferred onto each agar plate where every root samples were replicated using three medium agar plates. In total, 180 medium agar plates were inoculated with banana root samples while 60 medium plates were inoculated with oil palm roots samples to isolate both the NFB and PSB. The inoculated NFB medium agar plates were incubated at room temperature for 48 hours where bacterial colonies formed on the nitrogen-free NFB agar medium were termed as NFB [4]. Similar procedures were used for the isolation of PSB on the PKV medium agar plates, with the one-week incubation period being the only difference. Bacterial colonies that developed a clear zone surrounding its colonies were considered positive in phosphate solubilizing activity, hence termed as phosphate-solubilizing bacteria [3]. The bacterial colonies that showed positive response on NFB and PKV medium were transferred onto fresh nutrient agar (NA) medium (23 g/L, Merck) for further isolation. Continuous sub-culturing onto fresh NA mediums was carried out until a single pure bacterial colony was obtained. The pure bacterial colonies were grown on slant NA media for further study.

2.3. Screening of Nitrogen Fixing and Phosphate Solubilizing Bacterial Isolates

The nitrogen fixing bacteria and phosphate solubilizing bacterial isolates that were obtained from banana and oil palm root samples were further inoculated onto both BNF and PKV medium agar to further establish its nitrogen fixing and phosphate solubilizing capacities. After that, isolates showing consistent nitrogen fixing and phosphate solubilizing properties were selected and determined for the extent of its nitrogen fixing and phosphate solubilizing capabilities. For the determination of Nitrogen Fixation Capability, one loopful of the selected bacterial isolates (identified as having nitrogen fixing activity) grown on NA slant agar were transferred into a series of 500 ml Erlenmeyer flasks containing 100 ml of NB (8 g/L, Merck) followed by shaking at 160 rpm (SSL1, Stuart) for 24 hours at 28°C. After that, 10 ml of culture broth was transferred into 100 ml of BNF media in 500 ml Erlenmeyer flask and shaken at 160 rpm for 24 h at 28°C. Three replications were carried out for all bacterial isolates. At the end of the incubation period, the culture broth was filtered and subjected to Kjeldahl digestion to determine the total nitrogen content [5]. Total nitrogen content was expressed as percentage of the sample:

$$\text{Volume of sample solution} = [(V_1\text{-}V_2)\text{ ml} \times \text{Molarity of NaOH}]/\text{ W} \times [(\text{Volume of sample solution}/\text{ Volume of sample taken} \times 0.014 \times 100)/\text{ W}$$

where V_1 = volume of 0.1M NaOH used to neutralize excess 0.1M HCl in the blank determination, V_2 = volume of 0.1M NaOH used to neutralize excess 0.1M HCl in the sample titration, W = volume of sample taken for analysis. The phosphate solubilizing activity was carried out by transferring one loopful of the selected bacterial isolates

(identified as having phosphate-solubilizing activity) onto the National Botanical Research Institute Phosphate (NBRIP) agar medium. Four isolates per plate were inoculated in triplicate on NBRIP agar medium by followed by incubation at 25°C. The diameter of colony growth and diameter of clear halo zone were measured after 14 days of incubation. The ability of the isolates to solubilize insoluble phosphate was determined by the Solubilization Index (SI) which is defined as the ratio between the total diameter (colony + halo zone) to the colony diameter [6].

2.4. Growth Profile of Selected Bacterial Strains and its identification

Five selected isolates of NFB (N15, N14, N13, N12, N7) and three selected isolates from PSB (P7, P6, P3) were evaluated for its growth profile and viable cell count. The selection was made based on isolates showing consistent nitrogen fixing and phosphate solubilizing properties. Bacterial isolates with the fastest growth rate and highest viable cell count will be identified and used in subsequent biofertilizer formulation work. The isolates were prepared on nutrient slant agar and sent to First BASE Laboratories Sdn Bhd, Seri Kembangan, Selangor for identification using 16S rRNA gene sequencing.

2.5. FESEM Evaluation on Root Colonization Ability of the Isolated NFB and PSB

The potential root colonization ability of the bacterial isolates were evaluated using the following methods; a series of polyethylene bags containing 130g of EFB compost (C/N ratio <20, moisture content 50%, 1-2mm, autoclaved (121^{o}, 20min, 3 consecutive days) was injected with 70mL of individual bacterial inoculant (final moisture content around 35%). The EFB compost containing bacteria was then used to evaluate the growth of 3 weeks banana tissue culture seedlings and germinated oil palm seeds under glass house (100' length x 30' width x 20' height) for 16 weeks. The fresh root samples were prepared to observe the bacterial colonization on the root surface at 0, 4, 8, 12 and 16 weeks after planting. All samples were dried for 24 hours using a desiccator prior to viewing under Field Emission Scanning Electron Microscope, FESEM (JSM6701F, JEOL) at the Material Science Laboratory, Faculty of Mechanical Engineering, UTM, Johor Bahru.

2.6. The Experimental Design and Data Analysis

The experiment was conducted in Complete Randomised Design (CRD) with 3 replications for each treatment where single plant represent one replication. The banana seedlings and oil palm seedling germinated seeds were planted on the plastic pot containing 0.5 kg of growing media. Analysis of variance (ANOVA) was used to test the treatment effects and means of treatment were compared through Duncan's Multiple Range Test (DMRT) by using the Statistical Analysis System (SAS) version 9.1 software.

3. Results

3.1. Isolation of Potential Nitrogen Fixing Bacteria and Phosphate Solubilizing Bacteria from Banana and Oil Palm Roots

A total of 42 isolates of potential phosphate solubilizing bacteria (PSB) and 30 isolates of potential nitrogen fixing bacteria (NFB) were isolated from banana root sample. However, only a single isolate each of potential PSB and NFB were isolated from oil palm root

sample due to high contamination. The potential PSB isolates consisted of four major groups based on colour morphology namely red, yellow, cream and cream translucent. Meanwhile, the potential NFB isolates consisted of five major groups which were red, orange, yellow, cream and cream translucent. The potential isolates, which have the potential to carry out both phosphate solubilizing and nitrogen fixing activities, were red and cream translucent. Both of these isolates (NFB and PSB) were further selected based on their colour morphology and minimum 1 isolate were selected from each morphological group to represent each sample sources for banana. Therefore, from the banana root samples, only 14 isolates of potential NFB (N1-N14) and 12 isolates of potential PSB (P1-P16) were selected for subsequent studies. For bacterial isolates originated from oil palm root samples, both isolates that grow well on BNF agar media (N15) and PKV agar media (P17) were chosen for further study.

All 15 isolates for potential NFB and 14 potential isolates for PSB were re- inoculated onto both Burk nitrogen free (BNF) and Pikovskaya (PKV) agar media to re-assess their abilities in nitrogen fixation and phosphate solubilization as well as determining isolates that could have both the NFB and PSB functions. The re- inoculation result indicated that all the 13 potential PSB isolates showed positives response by developing clear halo zone around the bacteria colony with six isolates (P2, P3, P7, P10, P11 and P16) showing strong response i.e better growth, as indicated from the larger halo zone (Table 1). For potential NFB, all 15 isolates showed positive growth on BNF agar medium with six isolates (N1, N2, N3, N10, N11 and N12) showing stronger response with rapid growth.

It is worth noting that from the nine potential PSB isolates showing positive growth on BNF agar media, four isolates namely isolate P7, P8, P11 and P16 showed better adaptability to the BNF agar media as indicated by its rapid growth compared to the other five isolates. Meanwhile, nine potential NFB isolates showed positive response on PKV agar media based on the development of clear halo zone surrounding the bacteria colony, of which seven isolates (N1, N4, N7, N8, N9, N12 and N15) showed stronger response with bigger halo zone. Based on this, isolates showing better adaptability to grow in BNF (P7, P8, P11, P16) and PKV agar media (N1, N4, N7, N8, N9, N12, N15) were selected for further studies.

The bacterial colonies that grow rapidly on NFB agar medium and developed a clear halo zone around the colonies on PKV agar media was isolated and sub-cultured on nutrient agar media plate to obtain pure bacterial colonies. From the results obtained from the growth profile and determination of viable cell count studies, three isolates with the highest to moderate-fast growth capacity namely isolate N15, N12, and N7 were selected as the final isolates for mixed cultures evaluation. Isolate N15 was determined as the best isolate with strong capacity to carry out both NFB and PSB features. However, it grows moderately fast. Isolate N7 showed strong ability as PSB, moderate NFB but grew fastest among all of the isolates. Isolate N12 is an excellent NFB with low PSB capacity and grows moderately fast growth.

Table 1. Profile on the response of isolates on BNF and PKV agar media

Potential PSB isolates	**PKV**	**BNF**	**Potential NFB isolates**	**BNF**	**PKV**	**Potential PSB & NFB isolates**	**PKV**	**BNF**
P1	+	-	N1	++	++	P7	++	++
P2	++	+	N2	++	-	P8	+	++

P3	++	-	N3	++	-	P11	++	++
P5	+	+	N4	+	++	P16	++	++
P6	+	+	N5	+	+	N1	++	++
P7	++	++	N6	+	-	N4	++	+
P8	+	++	N7	+	++	N7	++	+
P9	+	+	N8	+	++	N8	++	+
P10	++	-	N9	+	++	N9	++	+
P11	++	++	N10	++	-	N12	++	++
P15	+	+	N11	++	-	N15	++	+
P16	++	++	N12	++	++			
P17	+	-	N13	+	+			
			N14	+	-			
			N15	+	++			

Negative response; + Positive response; ++ Strong response

3.2. Nitrogen Fixing Capacity of Selected Isolates

All potential NFB isolates (N1-N15) including 4 selected PSB isolates (P7, P8, P11, P16) that showed strong growth on Burk nitrogen free (BNF) agar media were evaluated for its nitrogen fixing capacity. Analysis of nitrogen content in BNF broth medium, after 24 hours of inoculation with the isolated cultures, indicated that among the 15 NFB isolates, N15, N14, N13 and N12 promises to be excellent NFB with highest nitrogen content of 150.4 ppm, 148.3 ppm, 144.6 ppm and 143.1 ppm respectively. These values were significantly different as compared to control i.e. without inoculum, C1. Meanwhile, 6 potential NFB isolates N8, N4, N7, N3, N10 and N2 showed moderate level of nitrogen content at 93.9 ppm, 90.2 ppm, 87.6 ppm, 86.1 ppm and 72.6 ppm respectively. Isolate N9 showed low level of nitrogen content at 61.7 ppm. However, the nitrogen-fixing capacities of isolate N9 (nitrogen content of 61.7 ppm), N1 (56.5 ppm), N6 (55.5 ppm), N5 (48.8 ppm) and N11 (48.2 ppm) were not significantly different (Tukey 5%) as compared to control culture (without inoculum) with 28.5 ppm of nitrogen content. The nitrogen fixing capacity evaluation for the 4 selected PSB isolates indicated isolate P7 was moderate in nitrogen fixing capacity with the nitrogen content of 77.8 ppm and isolates P8 was low in nitrogen fixing capacity with the nitrogen content of 69.5 ppm. The other 2 isolates P11 and P16 with 59.6 ppm and 38.9 ppm of nitrogen level respectively were not significant different (Tukey 5%) as compared to control culture (without inoculums) with 28.5 ppm of nitrogen content. Based on these results, isolates N15, N14, N13 and N12 (NFB) and isolate P7 (PSB) were selected for further study in biofertilizer formulation.

3.3. Phosphate Solubilizing Activity of Selected Isolates

Isolates P3 and P6 were among the potential PSB that showed highest phosphate solubilizing activity with the SI of 2.87 and 2.49 respectively. Isolates P11, P16, P7, P8, P2, P10 and P1 were moderate in phosphate solubilizing activity with the SI of 2.46, 2.31, 2.25, 2.19, 2.19, 2.02 and 2.01 respectively. However, isolates P9, P15, P5 and P17 were lowest in phosphate solubilizing activity amongst the potential PSB isolates. Meanwhile, the 2 selected NFB isolates N15 and N7 showed moderate capacity in phosphate solubilizing activity with SI of 2.40 and 2.15 respectively. The other 5 isolates (N12, N9, N1, N4 and N8) were categorized as having low phosphate solubilizing activity with SI values of 1.91, 1.87, 1.85, 1.70 and 1.61 respectively.

3.4. Selection of Potential Nitrogen Fixing and Phosphate Solubilizing Bacteria

Four isolates (N15, N12, N7 and P7) were determined to have the ability to function as both NFB and PSB, two isolates (N13 and N14) as NFB and another two isolates (P3 and P6) as PSB. All isolates were evaluated for its growth profile and viable cell count at 24, 36 and 48 hours, acting as another parameter to further screen the isolates prior to final study on biofertilizer formulation (Table 2). Isolates N7, N12, N15, P3 and N14 showed good early growth with absorbance reading of 0.292, 0.042, 0.016, 0.013 and 0.009 respectively after 8 hours of inoculation period. These 5 isolates showed similar increasing trend for cell concentration (after 12 hours of incubation) with absorbance values of 0.847 for N7, 0.716 (N15), 0.690 (N12), 0.516 (N14) and 0.475 (P3). Meanwhile, isolates P7, N13 and P6 seem to be growing at a slower rate at the earlier stage of growth in NB with significant growth only recorded after 12 hours of inoculation with absorbance reading of 0.320, 0.139 and 0.013 respectively. However, these 3 isolates showed higher cell concentration among the isolates at 24 hours after inoculation, where P6 and P7 recorded highest absorbance reading at 1.266 and 1.205 followed by isolates P3, N13, N7, N15, N12 and N14 with the absorbance reading of 1.139, 1.118, 1.068, 1.031, 0.966 and 0.908 respectively. The cell concentration of all the isolates in NB was almost similar at 24 hours and 48 hours after inoculation. Isolates N7, N15 and N12 were clearly demonstrated as the fast growing isolates than the others from its higher amount of cell concentration at 8 and 12 hours after inoculation. The first 4 hours after inoculation was the lag phase for all the isolates. The exponential phase for Isolate N7, N12, N15, P3 and P14 started after 8 hours of inoculation that stretches until 24 hours. Whereas, a much later exponential phase (after 12 hours) were observed for isolates P6, P7 and N13 only. All the isolates seem to be in stationary phase at 24 hours until 48 hours after inoculation. Isolates P6 and N15 were among the isolates that produced highest viable cell at 24, 36 and 48 hours after inoculation in nutrient broth, they consisted about 6.33-7.37 x 10^9 CFU/mL and 3.53-3.97 x 10^9 CFU/mL respectively at the stationary phase. Isolates P3, N7 and P7 consisted moderately of viable cell with 7.63-8.43 x 10^8 CFU/mL, 5.07-5.77 x 10^8 CFU/mL and 4.87-5.83 x 10^8 CFU/mL respectively, followed by isolates N14, N12 and N13 that consisted lower viable cell in stationary phase with 2.53-2.87 x 10^8 CFU/mL, 2.47-2.80 x 10^8 CFU/mL and 1.64-2.04 x 10^8 CFU/mL respectively.