Identification of Nitrogen Fixing Bacteria from Indigenous Microorganisms (IMO) Isolated From Bamboo Area at Taman Pertanian and Lepar Hilir, Pahang

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Abstract

Agricultural sector in Malaysia utilises high amount of chemical fertilizers as well as organic manures secreted by animals. The utilisation of the fertilizers in agricultural sector increases the level of environmental pollution. The usage of chemical fertilizers can be reduced by applying indigenous microorganisms (IMO) as beneficial microorganisms that are able to provide sufficient nutrients to various crops in agricultural sector. In this study, IMO that has been isolated from bamboo area at Taman Pertanian, Pahang and Lepar Hilir, Pahang were subjected to single colonies isolation and nitrogen fixing bacteria were screened by using several biochemical approaches including gram staining, carbohydrate fermentation and nitrate reduction tests. From the screening, a total of six bacterial isolates have been identified as potential beneficial microorganisms to fix free nitrogen to nitrate, as one of important nutrient for the growth of crops. All the six bacterial isolates were further subjected to genomic DNA isolation and followed by PCR amplification of 16S rRNA gene using 27F and 1492R primers. The PCR-amplified products were purified and further sequenced using the same primers. DNA sequence of each 16S rRNA gene of all the bacterial isolates were subjected to BLASTN analysis at the NCBI GenBank database. Interestingly, the BLASTN analysis has shown that all the bacterial isolates are homologous to strains of *Bacillus* spp. including *B. subtilis, B. amyloliquefaciens* and *B. tequilensis,* respectively. Phylogenic tree analysis on rRNA genes of all the bacterial isolates has shown their closely-relatedness with other strains of *Bacillus* spp. In conclusion, Bacillus spp. including *B. subtilis, B. amyloliquefaciens* and *B. tequilensis* isolated from the selected bamboo areas have potential to be utilised in Malaysia agricultural sector as nitrogen fixing bacteria that might reduce the usage of chemical fertilizers.

Keywords: Microorganisms, Indigenous Microorganisms (IMO), Nitrogen Fixing bacteria, Bacillus

1. INTRODUCTION

Technology of Indigenous Microoragnisms (IMO) was developed by Dr. ChouHankyu in 1960s. Generally, IMO and Effective Microorganisms (EM) are same. The term "IMO" refers to a group of beneficial bacteria that are locally isolated, thus it name indigenous. On the other hand, EM is man-made, which is laboratory-cultured mixture of microorganisms (Lakshman and Sai, 2015). It was suggested IMO is mainly consists of *Lactobacillus,* nitrogen-fixing bacteria, actinomycetes, photosynthetic bacteria, and yeast. The effectiveness of IMO brings effects on the soil by altering the physico-chemical, biological and enzyme properties of the soil as it has adapted to the local environment and survived. According to Sumathi, et al. (2012), IMO has wide range of naturally occurring plant nutrients and trace elements, carbohydrates, amino acids, and the plant's growth promoters where all of these can act as soil conditioner or enhancer by activating the microbial activity in the soil.

In the past, when IMO application into soil has shown the gradual increase of nitrogen-fixing bacteria (Higa T and Wididana 1991). Generally, nitrogen-fixing bacteria are active in soil zone in the presence of plants' roots. This type of bacteria has potential to transform free atmospheric nitrogen to organic compounds either independently or in symbiosis with plants. Additionally, it can assimilate nitrogen into organic compounds that are readily digested or absorbed by plants as nutrient sources (Bakulin et al., 2007). There are many genus of bacteria that are categorised as nitrogen-fixing bacteria including *Azospririllum, Azotobacter, Enterobacter* which have been found in rhizosphere of various plants including sugarcane, maize, wheat, rice and grasses (Affourtit et al., 2011). Subsequently, these bacteria enable of fixing atmospheric nitrogen, solubilize phosphorus and iron, and enhance production of plant hormones.

2. MATERIALS AND METHODS

In this study, the experiment was carried out in bamboo bushes at Taman Pertanian, Pahang and Lepar Hilir, Pahang to isolate nitrogen-fixing bacteria. One kilogram of steamed rice was filled into each container. The containers were covered with white cotton cloth and additional cover was made up using fallen leaves from the harvest location. The containers were left undisturbed for a minimum of 4 to 5 days to allow fermentation of the steamed rice. After formation of white molds, one kilogram of brown sugar was added as carbon source to increase fermentation process of the steamed rice for formation of IMO mixture. Then, 200 grams of coffee grounds or one kilogram of banana peels were mixed with the samples, respectively, as nitrogen source. Once the fermentation completed, the ferments were transferred into two liter Schott bottles and labelled accordingly as shown in Table 1.

Table 1. Formulations for six types of IMOs.

Label	Type of IMO	IMO Formulation
CL-LH	Control IMO from Felda Lepar Hilir	1 kg steamed rice + 1 kg brown sugar
CL-TP	Control IMO from Taman Pertanian	1 kg steamed rice + 1 kg brown sugar
CG-LH	Coffee ground IMO from Felda Lepar Hilir	1 kg steamed rice + 1 kg brown sugar + 200 g coffee ground
CG-TP	Coffee ground IMO from Taman Pertanian	1 kg steamed rice + 1 kg brown sugar + 200 g coffee ground
BP-LH	Banana peels IMO from Felda Lepar Hilir	1 kg steamed rice + 1 kg brown sugar + 1 kg banana peels
BP-TP	Banana peels IMO from Taman Pertanian	1 kg steamed rice + 1 kg brown sugar + 1 kg banana peels

Legend: LH: Lepar Hilir; TP: Taman Pertanian; CL: Control; CG: Coffee Ground; BP: Banana Peels

Identification on groups of the microorganisms in the isolated IMOs was further performed using plates containing Jensen's medium as a selection media for isolation of nitrogen-fixing bacteria. The plates were then incubated at appropriate temperature and growth of the bacteria was observed and recorded.

A series of biochemical tests were carried out to identify the species of each bacterial isolate based on their enzymatic reaction and responses towards biochemical reactions. To distinguish the nitrogen-fixing bacteria, three types of biochemical test were performed including gram staining, carbohydrate fermentation test, and nitrate reduction test.

In addition, further confirmation on the species identification was carried out using 16S rRNA analysis. Genomic DNA was extracted from each bacterial isolate, PCR-amplified using specific primers for 16S rRNA gene and followed by single pass DNA sequencing on the PCR-amplified products. Sequence of 16S rRNA gene of each bacterial isolate was further subjected to BLASTN analysis by comparing to their homologous sequences that are available at the NCBI GenBank database. Genetic relationship between all the isolates was determined by using a phylogenetic tree that has been constructed using neighbor-joining method.

3. RESULTS AND DISCUSSION

The bacterial colonies of nitrogen-fixing bacteria that grown on Jensen's medium were observed and classified based on their shape, surface, edge and elevation. From the observation, all the bacterial isolates were in circular shape with smooth and shiny surfaces. The edge of colonies was perceived to appear as an entire edge and the elevation of colonies of nitrogen-fixing bacteria was in pulvinate. Figure 1 shows the illustration of nitrogen-fixing bacteria in laboratory.

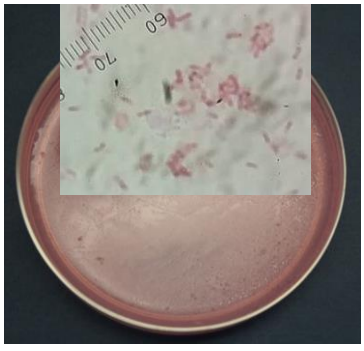

Figure 1. The illustrate of Nitrogen-fixing bacteria in laboratory

Next, a series of biochemical tests were carried out for species identification. Gram staining on all the bacterial isolates has shown their Gram-negative bacterial characteristic as they are observed in pink colour. In general, the cell wall of gram-negative bacteria is made up of several layers of peptidoglycan. These layers of peptidoglycan will be disturbed as the Gram reagent introduced. Thus, the cells will be decolorized and stained red with safranin (Beveridge and Graham, 1991). For carbohydrate fermentation test, all the bacterial isolates have shown positive result. For all the bacterial isolates, it has been observed that methyl red pH indicator changed from red to yellow and gas was

trapped in the Durham tubes. Durham tubes were put into the culture broth to detect the presence of acid gas during fermentation (Reiner, 2013). It means that the nitrogen-fixing bacteria are able to utilise different types of sugar for their fermentation processes. Meanwhile, nitrate reduction test was carried out to determine the capability of all the bacterial isolates for production of nitrate reductase and nitrite reductase. These two enzymes were selected in this study due to their involvement in reducing nitrate to free nitrogen gas (Jimienez et al., 2011). In this study, all the bacterial isolates have shown a positive result whereby nitrate has been detected to reduce to free nitrogen gas. Based on gram staining and the biochemical test, all the bacterial isolates could be classified as Nitrogen-fixing bacteria due to their gram negative mobile rods that are are stained in pink (Figure 1) as well as their positive result on the nitrate reduction test.

In molecular analysis on 16S rRNA gene of all the bacterial isolates, a total of twelve partial sequences (1, 500 bp) of bacteria genes were aligned, including one sequence of *Tropheus moorii* mitochondrion gene for cytochrome b as outgroup. The neighbor joining (NJ) tree (Figure 2) revealed that the bacterial isolates were classified based on genus and species. From the phylogenetic tree, bacterial isolates A1, A3, A6, B1, B2, B3, B4, B5, and B6 were resolved under level genus *Bacillus sp.* only. This is because of the species involved is rare type, so there is little information in database GenBank that is not enough to describe the species of bacteria involved. Other species, species A2, A4, and A5 were resolved under species level. This is because database in GenBank have enough information to describe the species of bacteria. Based on the phylogenetic tree, bacterial isolate A2 is homologous to *B. Tequilensis* strain SH145 while bacterial isolate A4 and A5 are homologous to *B. tequilensis* strain xuru17 and *B. amyloliquefaciences* strain SN-23, respectively.

4. CONCLUSIONS

From this study, all the bacterial isolates are homologous to strains of *Bacillus* spp. including *B. subtilis*, *B. amyloliquefaciens* and *B. tequilensis*, respectively. Phylogenic tree analysis on rRNA genes of all the bacterial isolates has shown their closely-relatedness with other strains of *Bacillus* spp. Bacillus spp. including *B. subtilis*, *B. amyloliquefaciens* and *B. tequilensis* isolated from the selected bamboo areas have potential to be utilised in Malaysia agricultural sector as nitrogen fixing bacteria that might reduce the usage of chemical fertilizers.

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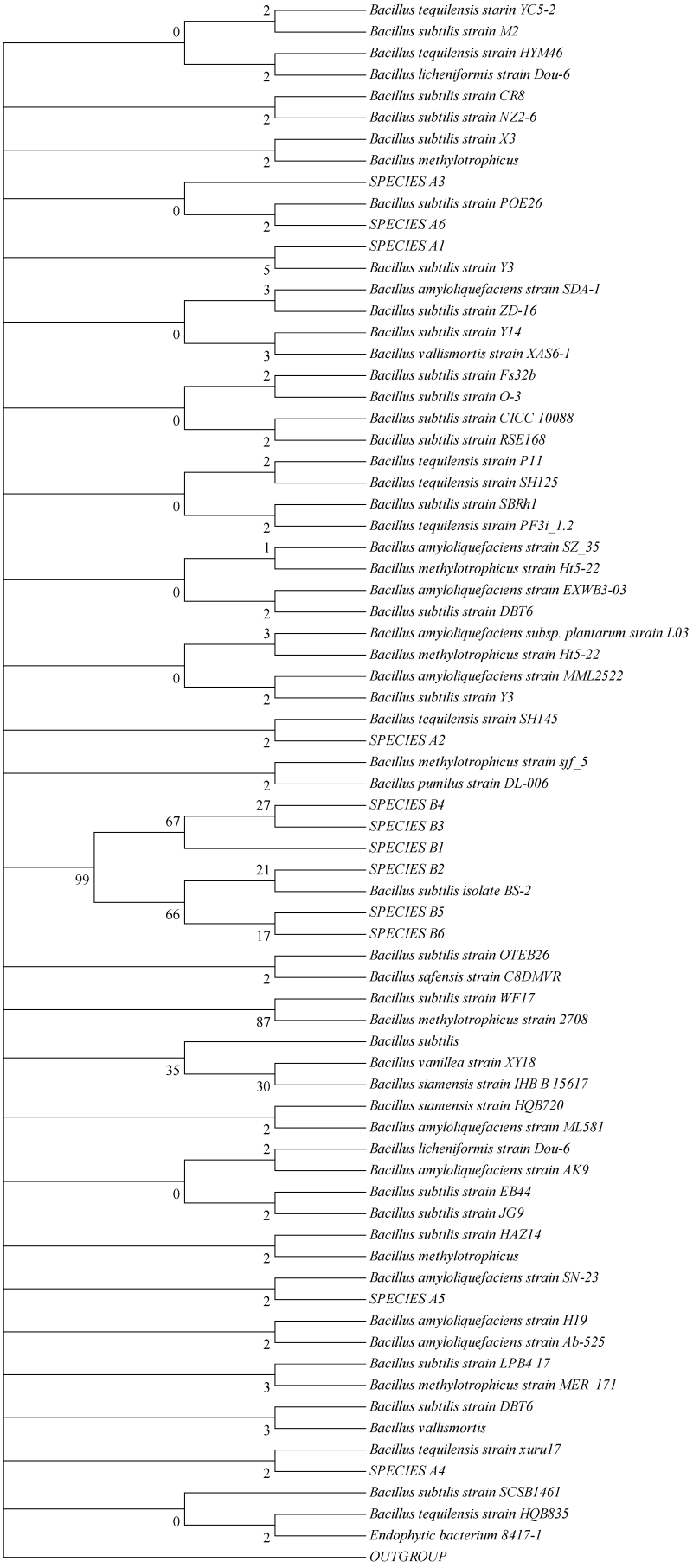

Figure 2. The Evolutionary of Taxa Bacteria