International Journal of Engineering & Technology, 7 (4.43) (2018) 140-144

SPC

International Journal of Engineering & Technology

Website: www.sciencepubco.com/index.php/IJET

Short communication

Determination of Nitrogen Fixing Capacity of Bacteria Isolated from the Rhizosphere of Acacia Mangium from the BRIS Soil of Tembila, Besut, Terengganu, Malaysia

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Abstract

The present study was conducted to isolate beneficial bacteria with nitrogen fixing potential from the rhizosphere of *Acacia mangium* plant. The plant grows wildly on the untouched area of problematic BRIS soil in Tembila, Terengganu. A total of 24 bacterial isolates were successfully sequestered from the soil samples using the Burk's nitrogen free medium. Based on the preliminary morphology screening, eight bacterial isolates were selected and their morphological and biochemical characteristics were recorded. Three bacterial isolates namely UA1, UA6 and UAA2 were selected as final species for antibiotic sensitivity test and Acetylene Reduction Assay (ARA). All three bacterial isolates showed a high amount of nitrogenase activity in ARA with UA6 produced the highest and significant amount of ethylene with 18.55 $nmolC_2H_4mL^{-1}h^{-1}$. UA1 and UAA2 showed almost similar amount of ethylene with 17.10 and 17.00 $nmolC_2H_4mL^{-1}h^{-1}$ respectively. The isolated bacteria characteristics through all the test showed that these bacteria have potential to be explore for biofertilizer production industry.

Keywords: *ARA; bacteria; biofertilizer BRIS soil; nitrogen; rhizosphere.*

1. Introduction

Nitrogen is the most crucial nutrient for plant growth and development. Nitrogen is freely available in the biosphere in the form of atmospheric nitrogen but it cannot be directly utilised by the plant. It can only be made available to plants by the process of Biological Nitrogen Fixation (BNF) [1]. In this process, atmospheric nitrogen is converted into ammonia by nitrogen fixing bacteria or rhizobia in the presence of specific enzyme known as nitrogenase. The mechanism could either be in a symbiotic manner with the plant such as in the rhizobium and leguminous plants interaction or in non-symbiotic or free-living forms [2]. Non- symbiotic nitrogen fixation can only be carried out by selected microorganisms, mostly blue green algae and free-living bacteria [3].

Plant growth and productivity is highly influenced by soil minerals especially nitrogen. Sandy beach ridges can be found in the Kelantan - Terengganu Plains. This soil has a sand texture named as Beach Ridges Interspersed with Swales (BRIS) soil. The swales are found in the depression areas between the ridges and swamped by water for most part of the year [4]. The beach ridges has sandy soil of more than 95 % sand with are usually composed of coarse sand fraction in the topsoil and extremely fine sand in the subsoil. The high sand contents results in loss of moisture and water retention capacity as a consequence of excessive drainage. BRIS soil also has limited ability to support plant growth because of its nutrient deficiency caused by high drainage [5]

Plant biodiversity in BRIS soil area are generally low due to its obstructive characters which include low water retention capacity, low nutrient contents and comparatively high temperature [6]. Observation of BRIS soil in Tembila area found that most of the area are covered by grasses and small shrubs with most of the untouched areas are dominated by *Acacia mangium*. Geographically, plant grows behind mangroves in seasonal swamps, along streams and on well-drained flats, low ridges and mountain foothills [7]. It inhabits humid, tropical lowland zones that tolerates to the pH levels between 4.5 and 6.5. Mangium belongs to the sub-family Mimosoideae of Leguminosae family. It is native to Australia, Papua New Guinea and Indonesia and was first introduced to Sabah, Malaysia in 1967 [8]. A shortage of sawlogs for the production of general utility timber before the end of the 19th century has caused this species to be planted across Peninsular Malaysia mainly in the states of Johore, Negeri Sembilan, Pahang and Selangor with the total area of 51 768 Ha [9]. At present, *A. mangium* plantation does not exist in Terengganu but the plant grows wildly in various places including along the Terengganu plains BRIS soil area.

BNF is indispensable in both ecological and agricultural point of view. BNF by association of free living microorganisms possess a great practical importance because the use of chemical nitrogenous fertilizers has been causing high levels of environmental pollution. The use of excessive nitrogen-based fertilizer has caused serious water pollution and eutrophication of

lakes and rivers [10]. Therefore, the isolation and characterization of microorganisms with the ability to fix nitrogen has become crucial to alleviate these problems. Through analysis of the newly isolated bacterial strains, the nitrogen-fixing capability for nitrogen source can be utilized for application like biofertilizer. This study aims to isolate bacterial strains from the rhizosphere of wild *A. mangium* at the BRIS soil area in Tembila, Terengganu to determine their ability to fix atmospheric nitrogen using acetylene reduction assay.

2. Materials and Methods

2.1. Isolation of Nitrogen-fixing bacteria

Soil samples were collected around an *A. mangium* rhizosphere at Besut Campus, UniSZA (5°45'26.4"N 102°37'21.3E). Five samples were randomly obtained from the rhizosphere at 10-15 cm depth. The samples were air-dried and filtered through a 2 mm sieve before being mixed into a single composite sample. About 10 g of soil was suspended in 90 mL sterile saline water, rotary shaked at 150 rpm for one hour at room temperature (28 ± 2 °C) and serially diluted. Aliquots 50µl from dilutions 10^{-4} until 10^{-6} and inoculated on Burk's N-free medium (HiMedia) for three days at room temperature (28 ± 2 °C). The colonies that showed different morphology were isolated and pure colonies were obtained by repeated streaking on Burk's N-free medium [3].

2.2. Morphology Characterization

The pure isolates of BRIS soil bacteria were characterized under a stereo microscope. The following traits were observed: colour pigment, form, elevation, margin, size, surface and opacity [11].

2.3. Biochemical Tests

Biochemical test for Gram staining, catalase, nitrate reduction, indole, and urease were carried out according to the method suggested by Cappucino and Sherman [12]. Determination of antibiotics sensitivity were performed using the Kirby-Bauer disk diffusion method [13]. The antibiotic discs that were separately diffused with chloramphenicol, rifampicin, penicillin, tetracycline, gentamycin and streptomycin were tested on the isolates. Plates containing bacterial cultures and discs were incubated at 37 °C overnight. Bacterial susceptibility was determined by measuring the size of inhibition zone showed around the disc which was interpreted by referring to the CLSI M100-S27 (Breakpoints and Interpretive Categories).

2.4. Acetylene Reduction Assay (ARA)

The isolated bacterial BNF was estimated singly and in mixed culture using the acetylene reduction assay method (ARA) adapted from Hardy et al. [14] and Somasegaran and Hoben [15]. The following formula was used to determine the amount of nitrogenase activity [16]: $N = (h\,x \times C \times V) / (hs \times 24.\ 9 \times t)$, where, N = the concentration of C_2H_4 (nmol ml^{-1} h^{-1}); $h\,x$ = the peak value of the sample: C = concentration of standard C_2H_4 (nmol $ml^{-1}h^{-1}$); V = volume of the vial; hs = peak value of C_2H_4; t = the time taken to complete a reaction (h).

3. Results and Discussion

A total of 24 bacterial isolates were successfully obtained from the composite samples. Aerobic bacteria were labelled as UA1-UA14 while anaerobic bacteria were labelled as UAA1-UAA10. As some of isolates showed identical morphology characters and Gram-staining results, only eight isolates were further subjected to the morphological and biochemical characterizations. The morphology and biochemical characteristics are described in Table 1 and 2.

It was observed that the forms of the colonies of bacterial isolates were mostly circular with some of them showed irregular form (Table 1). The surface of colonies were either moist or mucoid. Most of the colonies, which were selected visually based on differences under stereo microscope were of creamy white and red colour. The margin of the bacterial colonies were found to be entire and undulate. Also, most of the bacterial colony isolates had smooth and shiny texture with opaque and transparent opacity. Both the aerobic and anaerobic bacteria showed either Gram positive or Gram negative characteristics with cells being either coccus or rod in shapes. All eight isolates also had responded to the biochemical test with respective characteristics (Table 2).

Table 1: Colony morphology of bacterial isolates from the rhizosphere of *A. mangium* at BRIS soil area of Tembila, Terengganu.

Type	Color	Size	Form	Elevation	Margin	Texture	Surface	Opacity
UA1	creamy white	pinpoint	circular	flat	entire	smooth	moist	transparent
UA2	creamy white	pinpoint	circular	flat	entire	smooth	moist	transparent
UA3	creamy white	moderate	irregular	raised	undulate	shiny	mucoid	opaque
UA4	creamy white	large	circular	raised	entire	smooth	moist	opaque
UA5	creamy white	large	circular	raised	entire	smooth	moist	opaque
UA6	creamy white	large	irregular	raised	undulate	shiny	mucoid	opaque
UAA1	red	small	circular	convex	entire	shiny	moist	opaque
UAA2	creamy white	small	circular	convex	entire	shiny	moist	transparent

Table 2: Biochemical characteristics of bacterial isolates from the rhizosphere of *A. mangium* at BRIS soil area of Tembila, Terengganu.

Type	Gram	Cell shape	Catalase	Indole	Nitrate reduction	Urease
UA1	-	coccus	+	+	+	+
UA2	-	coccus	+	+	+	+
UA3	+	rod	+	+	+	+
UA4	+	rod	+	+	+	+
UA5	+	rod	+	+	+	+
UA6	+	rod	+	+	+	+
UAA1	-	rod	+	-	+	-
UAA2	-	rod	+	-	+	-

Based on combined morphology and biochemical characteristics, UA1 and UA2 can be assumed as a similar species, UA3, UA4, UA5 and UA6 as another species and UAA1 and UAA2 as another. Because of this, only UA1, UA6 and UAA2 isolates were selected to represent each species and tested for antibiotic sensitivity and Acetylene Reduction Assay. The zone of inhibition produced by the bacterial isolates against the chloramphenicol, rifampicin, penicillin, tetracycline, gentamycin and streptomycin is shown in Table 3. UA6 showed all positive response against the tested antibiotics by producing zones of inhibition. UA1 showed a negative result for Tetracycline and UAA2 showed a negative result for Rifampicin and Tetracycline.

Colony morphology and biochemical test were performed to characterized and identify the bacteria. It is because different species of bacteria are capable of producing distinct features of colonies [12]. The features of the colonies is crucial in bacterial identification and indispensable in the process of isolation and selection of bacteria of interest. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide to water and oxygen [17]. Catalase is very important to protect the cell from the harmful byproduct of many normal metabolic processes. Nitrate reductase is the enzyme that reduce nitrate (NO_3^-) to nitrite (NO_2^-) which may then again be degraded to various nitrogen products like nitrogen oxide, nitrous oxide and ammonia (NH_3). As nitrate is the predominant source of nitrogen in fertilized soils this reaction is critical for the production of protein in most crop plants [18]. Urease is the important enzyme in nitrogen metabolism that hydrolyses urea from fertilizers to ammonia and carbon dioxide to be assimilate by plants [19]. The indole test was conducted to determine the ability of the organism to convert tryptophan by tryptophanase into indole. A large number of Gram-positive and Gram-negative bacterial species are indole positive. Indole influences plant defense systems against herbivore attack, and promotes root and plant growth [20].

Table 3: Antibiotic sensitivity response of three selected bacterial isolates from the rhizosphere of *A. mangium* at BRIS soil area of Tembila, Terengganu.

Isolates	Antibiotics response / Zone of inhibition (cm)					
	Cam	Rif	Pen	Tet	Gen	Sm
UA 1	+ve (2.3)	+ve (1.4)	+ve (2.0)	-ve (0.0)	+ve (1.8)	+ve (2.0)
UA 6	+ve (3.4)	+ve (2.2)	+ve (2.1)	+ve (2.5)	+ve (2.7)	+ve (1.4)
UAA 2	+ve (2.4)	-ve (1.1)	+ve (2.3)	-ve (0.0)	+ve (2.1)	+ve (2.2)

Notes: Cam (chloramphenicol), Rif (rifampicin), Pen (penicillin), Tet (tetracycline), Gen (gentamycin) and Sm (streptomycin).

The BNF rate of the selected bacterial strains were quantified using ARA. A considerable amount of ethylene were produced by the selected strains after 1 h of incubation which ranged between 17.00- 21.63 nmolC_2H_4 $mL^{-1}h^{-1}$ (Fig 1). UA1 and UAA2 showed almost similar performance in BNF while UA6 showed the highest and significant BNF value of 18.55 nmolC_2H_4 $mL^{-1}h^{-1}$) among the three species. The mixed strains of UA1, UA6 and UAA2 showed the highest BNF value (21.63 nmolC_2H_4 $mL^{-1}h^{-1}$) compared by using single inoculum.

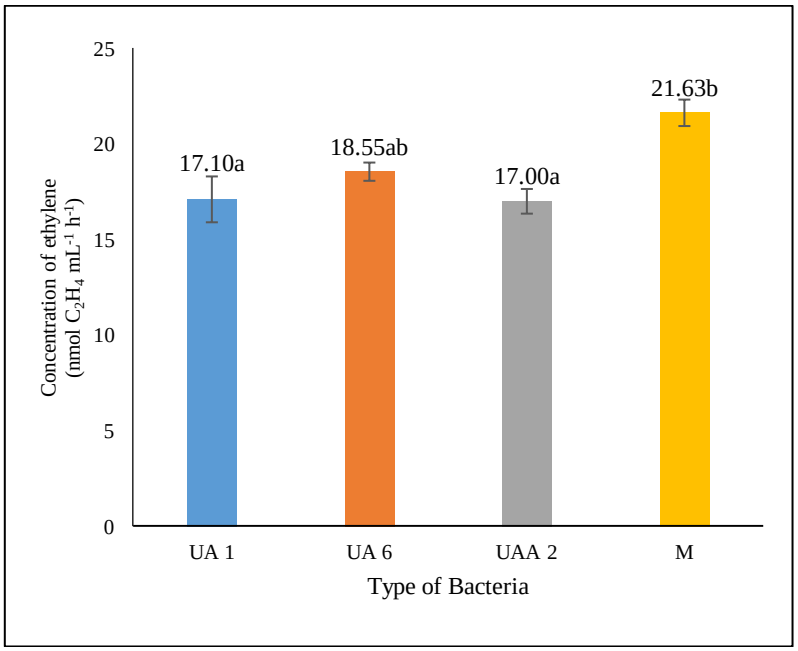

Fig 1: Acetylene reduction assay (ARA) of biological nitrogen fixation of selected bacterial strains. Means with different letters show significant difference at P<0.05 Tukey's multiple comparison, n=3. M refers to mixed isolates.

Extending application of BNF by any means is of huge importance. Various bacterial strains isolated from various places have been used in biofertilizer production. In Malaysia, mycorrhiza inoculums has been used in production of biofertilizer for supplying nutrient, amelioration of toxic effect in soils, root pest and disease control, improved water usage and soil fertility [21]. While another broad spectrum of *Azotobacter*, *Rhizobium*, *Azospirillum*, Blue Green Algae (BGA) and many more have been used in biofertilizer production [22]. Before any isolated bacteria can be used as a bio-inoculant in the production of biofertilizers, the characteristics of the bacteria need to be revealed. The plant growth promoting criteria of that bacteria also need to be identified. Knowledge of bacteria susceptibility to antibiotics is extremely important in the biofertilizer industry as it is to ensure that the bacteria are not capable of causing adverse effects on human health and environment. The minimum inhibitory concentration (MIC) of an organism is the lowest concentration of an antibiotic that will inhibit its growth [23]. The results of *in vitro*

antibiotic susceptibility tests is viable to predict the clinical response to treatment and guide the selection of antibiotics.

The amount of nitrogen fixed and transferred to their host plants vary greatly between species [24]. Several methods have been used to quantitatively and qualitatively measure BNF. ARA is among an established quantitative method that used worldwide for measuring BNF. This technique indirectly determine BNF by estimating enzyme activity based on the electron flux through nitrogenase. It involves a short term period under certain condition that useful as *in-situ* measurements of nitrogenase activity at a point in time. This method is not suitable for field evaluation as the assay only estimates the nitrogenase activity and does not refer to the amount of N_2 fixed which are incorporated into the plant [25]. However, the ARA method could still be useful in the preliminary screening of bacteria that can perform BNF prior to using other more complicated methods such as the 15N isotope technique.

ARA is an indirect method used to quantify the BNF rate since it measures the conversion activity of acetylene to ethylene by the nitrogenase enzyme that responsible for BNF by microorganisms. According to Watanabe et al. [26], ARA is considered positive if the activity is more than 6 nmol of C_2H_4 $tube^{-1}$, thus indicating the significance of the selected bacterial strains in this study. The ARA value obtained was in agreement with Różycki et al. [27] that reported the nitrogenase activity of diazotroph from the rhizosphere of pine (*Pinus sylvestris* L.) and oak (*Quercus robur* L.) were within the range from 4 to 20 nmoles C_2H_4 $culture^{-1}$ h^{-1}. However, the ethylene produced by the selected bacteria was slightly lower (0.59 – 8.89 %) than that indigenous species obtained from the rhizosphere of oil palm and soybean roots at the Selangor, Malaysia [28]. Nevertheless, the ARA values were higher compared to the values from the diazotroph of sandy Siberian soil that recorded only 0.1-0.8 nmol C_2H_4 d^{-1} $vial^{-1}$ [29].

4. Conclusion

This study has successfully isolated three bacteria (UA1, UA6 and UAA2) from the *A.mangium* rhizosphere at BRIS soil area in Tembila, Terengganu. These isolates have responded differently to the biochemical test. They also showed some inhibition in response to antibiotic test and ARA test. The reasonable amount of ethylene has been produced by all bacteria strains in ARA test. The characteristics recorded by the isolated bacteria show the potential of that bacteria to be used in biofertilizer production.

Acknowledgements

The authors are thankful to the Institute of Agricultural Production and Food Innovation UniSZA (AGROPOLIS), Centre of Farm Management UniSZA (PPL), Faculty of Bioresources and Food Industry UniSZA (FBIM), Centre for Research Management, Innovation and Commercialization UniSZA (RMIC) for the Pre-Commercialization grant (RR 217) and Ministry of Higher Education Malaysia for the Knowledge Transfer Program- KTP Community grant KTP/Bil 003/16 (KTP-R5).

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