Isolation and Characterization of Diazotrophic Rhizobacteria of Oil Palm Roots

Azlin, C.O., Amir, H. G*. and Chan, L. K.

School of Biological Sciences, Universiti Sains Malaysia,
11800 Minden, Penang, Malaysia

ABSTRACT

Beneficial rhizobacteria were isolated from two different compartments of oil palm roots; the rhizosphere or rhizoplane and the inner root tissues. The root samples were collected from oil palm plantation at Felda Lepar 9, Temerloh Pahang (Block 17, Square 6) (soil pH 4.30; 10:25 0.01M $CaCl_2$). Identification of the isolates was conducted by classical biochemical and physiological tests. Acetylene Reduction Assay (ARA) test was also conducted to quantify the ability of the isolates to fix atmospheric N_2. Twenty-nine strains of rhizobacteria were isolated from root samples and were maintained aerobically on N-free solid media. Seven of the isolates were identified as Gram negative while the rest were Gram positive. The isolates were successfully identified as *Paenibacillus durus* (formerly *P. azotofixans*), *Paenibacillus polymyxa*, *Azospirillum lipoferum*, *Herbaspirillum seropedicae* and *Acetobacter diazotrophicus*. The N_2 fixation capacities of the isolates ranged from 7.0×10^{-12} to 1.0×10^{-8} mol C_2H_4/cfu/hour.

Keywords: Diazotrophic bacteria, Acetylene Reduction Assay, rhizosphere, rhizoplane, root tissues

INTRODUCTION

Nitrogen is an essential component of all life form and is a paradox of nature because it cannot be assimilated by plants unless it is reduced to ammonia by diazotrophic microorganisms (Galdagi *et al.*, 2002). Some microorganisms can fix atmospheric nitrogen through a process called Biological Nitrogen Fixation (BNF) and this is an important source of nitrogen for various metabolisms (Flores-Encarnación *et al.*, 1999). It can also supply the fixed N_2 to the associated host plants (Gerk *et al.*, 2000; Barbara and Thomas, 1998). The process involves nitrogenase enzymes that reduce gaseous nitrogen into ammonia (NH_3) and ammonium (NH_4^+) (Chatterjee *et al.*, 1997). The process can be analyzed by Acetylene Reduction Assay (ARA) which enables quantification of nitrogenase enzyme activity through reduction of acetylene gas to ethylene. The prospect is bright to apply these N_2 fixing isolates effectively as a biofertilizer and bioenhancer for economics crops like oil palm and rice in Malaysia. Application of these biofertilizer and bioenhancer is a new approach to farming and serve as an alternative strategy to avoid excess applications of mineral fertilizer to plants. Thus, the objectives of this experiment were; 1) to isolate beneficial diazotrophic rhizobacteria from oil palm roots, 2) to quantify the N_2 fixation ability of these isolates by Acetylene Reduction Assay (ARA) and 3) to study the nitrogen fixation activity under aerobic or microaerophilic conditions.

*Corresponding author

MATERIALS AND METHODS

Isolation of diazotrophic rhizobacteria from oil palm roots

The roots were surface sterilized with 25 mL 95% ethanol for 30 seconds and washed twice with 50 mL sterile distilled water for 30 seconds. The roots were placed within test tubes containing nitrogen free semisolid malate medium (Nfb medium) to isolate rhizobacteria from the rhizosphere and rhizoplane through the formation of pellicle after 72 hours of incubation (Roslina *et al.*, 1995). A loopful of pellicle was streaked onto N-free ATCC solid medium (Eskew *et al.*, 1977) and kept under aerobic condition at 30°C for 48 hours. The roots were surface sterilized again using 25 mL 95% ethanol for 30 seconds, followed by washing with 50 mL sterile distilled water for 30 seconds. The roots were crushed with knife and forceps and placed in 20 mL Nfb semisolid malate medium to isolate endophytic rhizobacteria. After 72 hours of incubation, a loopful of the pellicle was streaked onto N-free ATCC solid medium and kept under aerobic condition at 30°C for 48 hours.

Quantification of nitrogen fixation activity by Acetylene Reduction Assay (ARA)

The isolates were tested for N_2 fixation activity using Acetylene Reduction Assay (ARA) for both liquid and semisolid culture conditions. 1) Liquid culture conditions: Liquid culture of each isolate (10 mL) was placed into an air-tight bottle volume 64 mL, approximately 10% of the air from each bottle was removed using an air-tight syringe and replaced by purified acetylene gas (99.8%). The bottles were incubated for 30 minutes for the reduction of acetylene to ethylene by the isolates. At the end of

incubation, 2 mL of the gas mixture was withdrawn and transferred into a vacuum tube (Vacutainer™ 7 mL) (Somasegaran and Hoben, 1985). The ethylene concentration was assayed using G-300 Hitachi gas chromatography fitted with carboxen 1004 micro packed, 2m x 1/16 in stainless steel column and flame ionization detector (FID). Nitrogen was used as the carrier gas, had a flow rate of 3.5 kgf/cm^2 and the temperature was maintained at 165°C (column) and 230°C (injector and detector), respectively. The actual concentration of ethylene was determined based on a standard curve of ethylene concentration (µmol C_2H_4) and the peak area percentage. The nitrogen fixation activity (µmol C_2H_4/cfu/hour) was defined from the ethylene concentration recorded (µmol C_2H_4) and the viable cell numbers (cfu) of the isolates tested. 2) Semisolid culture conditions: The ARA test was carried out in an air-tight 30 mL universal bottle containing 10 mL Nfb semisolid malate media, inoculated with the respective isolates. The isolates were incubated in the Nfb semisolid malate media for 72 hours for pellicle formation before the acetylene gas was injected into the head atmosphere of the universal bottles (5% v/v) followed by gas incubation for 24 hours at 30°C (Elbeltagy *et al*., 2001). At the end of the incubation, 2 mL of the gas mixture was withdrawn and transferred into a vacuum tube before assaying for ethylene.

Phenotypic characterization of diazotrophic bacteria

The isolates were identified based on physiological and biochemical characterizations. The tests involved were Gram staining, KOH test (Arthi *et al*., 2003), endospore staining, oxidase, catalase, nitrate reductase, urea hydrolysis, Voges-Proskauer, starch hydrolysis, hydrogen sulfide and indole tests (Seldin *et al*., 1998; Mavingui *et al*., 1992).

RESULTS AND DISCUSSION

Twenty-nine strains of rhizobacteria were successfully isolated from three different compartments of oil palm roots grown at Felda Lepar 9, Temerloh Pahang. Fifteen strains have been successfully isolated from rhizosphere or rhizoplane of oil palm root and fourteen strains were isolated from inner root tissues (Table 1 and 2). The strains isolated from the inner root tissues were categorized as putative endophytes that form association with their host plants by colonizing the internal tissue (Muthukumurasamy *et al*., 2002).

The Gram positive isolates that showed positive results for catalase, starch hydrolysis, nitrate reductase but negative for indole and oxidase test could be identified as *Paenibacillus macerans* and *Paenibacillus polymyxa*. However, *P. macerans* was different from *P. polymyxa* since the former did not produce acetylmethylcarbinol in the Voges-Proskauer test (Seldin *et al*., 1983) (Table 2). Isolate E14 was suspected to be *P. polymyxa* capable of fixing nitrogen up to 9.608 x 10^{-9} (mol C_2H_4/cfu/hour (microaerophilic conditions) and 4.442 x 10^{-9} (mol C_2H_4/cfu/hour under aerobic conditions (Table 3). The result also showed that isolate R4 had similar characteristics to *Paenibacillus durus* as shown earlier by Seldin *et al.,* (1998) which were positive for catalase and Voges-Proskauer test and negative for oxidase and nitrate reductase test. Isolate R4 could successfully fix N_2 under aerobic condition at 1.711 x 10^{-5} (mol C_2H_4/cfu/hour and 2.161 x 10^{-9} (mol C_2H_4/cfu/hour under microaerophilic condition (Table 3).

Results on the biochemical tests of isolates E10 and R2 (Table 1 and 2) showed similar characteristics to type strains of *Azospirillum brasilense* or *Herbaspirillum seropedicae*. The isolates were able to form pellicle in N-free semisolid malate medium. Formation of subsurface pellicle in N-free semisolid malate medium was often taken as an evidence that *Azospirillum* spp. was present (Roslina *et al*., 1985). Isolate E10 showed similar characteristics with *Azospirillum* spp. (positive results for methyl red and Voges-Proskauer test) and possibly belonged to *A. lipoferum* capable of fixing N_2 under microaerophilic conditions (2.367 x 10^{-8} µmol C_2H_4/cfu/hour). Isolate R2 was tentatively grouped together with *Herbaspirillum seropedicae* that could successfully fix N_2 in aerobic and microaerophilic conditions at 1.332 x 10^{-7} and 1.757 x 10^{-10} µmol C_2H_4/cfu/hour, respectively. Earlier findings have shown that *Azospirillum* could fix N_2 under microaerobic conditions and was known to colonize roots while *Herbaspirillum* was an endophytic diazotroph (Kennedy *et al*., 2004, Muthukumarasamy *et al*., 1999). Muthukumarasamy *et al*., (1999) also reported that *Herbaspirillum seropedicae* could increase leaf N content and cane yields significantly. Han and New (1998) have shown that *A. lipoferum* had a higher average nitrogenase activity than *A. brasilense*, both in Nfb medium and in association with wheat roots with 79.9 nmol C_2H_4/ mg protein/ hour.

Isolates R6 and R12 showed similar characteristics with *Acetobacter (Glucona acetobacter) diazotrophicus* (Table 1) (negative for Gram reaction, oxidase and starch hydrolysis but positive for catalase and nitrogenase activity), which was isolated earlier by Muthukumarasamy *et al*. (2002) from sugarcane. Ability of the isolates to form pellicle in Nfb semisolid malate medium was also recorded together with the ability to fix nitrogen under aerobic and microaerophilic conditions. Formation of acid from D-glucose indicated that the isolates could produce acid from D-glucose, D-mannose, *i*-inositol, D-mannitol, D-arabinose and D-fructose. Isolates R6 and R12 were suspected to be *A. diazotrophicus* capable of fixing N_2 at 4.8 x 10^{-11} µmol C_2H_4/cfu/hour and 4.012 x 10^{-7} µmol C_2H_4/cfu/hour in aerobic condition and 6.598 x 10^{-12} µmol C_2H_4/cfu/hour and 1.187 x 10^{-8} µmol C_2H_4/cfu/hour in microaerophilic condition, respectively. *Acetobacter* is well-known as an aerotolerant diazotroph in which oxygen is instrumental for the generation of large quantities of ATP required for nitrogen fixation (Flores-Encarnación *et al*., 1999) and has been proven to contribute up to 60-80 % of sugarcane plant N requirement (equivalent to over 200 kg/N/ha/yr) from N_2 fixation process (Kennedy *et al*., 2004).

Table1: Physiological and biochemical characterizations of the isolates from rhizosphere of oil palm roots

TEST	Isolates														
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15
Gram Staining	-	-	+	+	-	-	+	+	+	+	+	-	+	+	+
Endospore stain	+	-	+	+	-	-	-	+	+	+	-	-	-	-	-
KOH test	+	+	-	-	+	+	-	-	-	-	-	+	-	-	-
N-free medium	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+
Methyl Red	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
Voges-Proskauer	+	+	+	-	+	+	-	+	+	-	+	+	+	+	+
Nitrate Reductase	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-
Starch Hydrolysis	-	+	-	+	-	-	+	-	+	+	-	-	+	-	+
Catalase	+	-	-	+	+	+	+	+	+	-	+	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea test	+	+	+	-	+	-	-	-	+	+	-	+	-	+	-
Pellicle formation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2: Physiological and biochemical characterizations of the isolates from inner root tissues of oil palm

TEST	Isolates													
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14
Gram Staining	+	+	+	+	+	+	+	+	-	-	+	+	+	+
Endospore stain	-	-	+	-	-	-	-	-	+	-	-	+	+	+
KOH test	-	-	-	-	-	-	-	-	+	+	-	-	-	-
N-free medium	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	+	+	+	+	+	+	-	+	-	+	-	-
Methyl Red	-	+	+	+	+	+	+	+	+	+	-	-	-	+
Voges-Proskauer	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate Reductase	+	+	+	+	+	+	-	+	+	-	+	-	+	+
Starch Hydrolysis	-	+	-	+	+	+	+	-	-	+	-	-	-	+
Catalase	+	+	+	+	+	+	+	+	+	-	+	+	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urea test	+	+	+	-	+	-	-	+	+	+	-	-	-	-
Pellicle formation	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 3: Nitrogenase activities (μmol C_2H_4/cfu/hour) by isolates from the rhizosphere and inner root tissues of oil palm

Root Compartments	Isolates	Identified Strains	umol C_2H_4/cfu/hour (Aerobic conditions)	μmol C_2H_4/cfu/hour (Microaerophilic conditions)
Rhizosphere	R1	Unidentified	2.755×10^{-8}	1.187×10^{-8}
	R2	*H. seropedicae*	1.332×10^{-7}	1.757×10^{-10}
	R3	Unidentified	6.65×10^{-8}	1.652×10^{-8}
	R4	*P. durus*	1.711×10^{-5}	2.161×10^{-9}
	R5	Unidentified	-	1.176×10^{-11}
	R6	*A. diazotrophicus*	4.8×10^{-11}	6.598×10^{-12}
	R7	Unidentified	-	3.884×10^{-10}
	R8	Unidentified	3.911×10^{-10}	2.761×10^{-10}
	R9	Unidentified	-	-
	R10	Unidentified	-	-
	R11	Unidentified	2.429×10^{-7}	2.418×10^{-7}
	R12	*A. diazotrophicus*	4.012×10^{-7}	1.187×10^{-8}
	R13	Unidentified	-	5.638×10^{-11}
	R14	Unidentified	-	4.148×10^{-11}
	R15	Unidentified	-	2.126×10^{-11}
Root Tissues	E1	Unidentified	-	6.200×10^{-8}
	E2	Unidentified	-	3.080×10^{-8}
	E3	Unidentified	-	3.409×10^{-8}
	E4	Unidentified	-	8.592×10^{-7}
	E5	Unidentified	-	6.994×10^{-9}
	E6	Unidentified	-	4.919×10^{-9}
	E7	Unidentified	5.354×10^{-10}	3.165×10^{-8}
	E8	Unidentified	-	1.228×10^{-7}
	E9	Unidentified	7.865×10^{-10}	7.776×10^{-9}
	E10	*A. lipoferum*	-	2.367×10^{-8}
	E11	Unidentified	-	4.023×10^{-8}
	E12	Unidentified	-	2.965×10^{-8}
	E13	Unidentified	3.286×10^{-10}	2.979×10^{-9}
	E14	*P. polymyxa*	4.442×10^{-9}	9.608×10^{-9}

CONCLUSION

The experiment had successfully isolated and identified the diazotrophic rhizobacteria from the rhizosphere (*Herbaspirillum seropedicae*, *Paenibacillus durus* and *Acetobacter diazotrophicus*) and from the inner root tissues (*Azospirillum lipoferum* and *Paenibacillus polymyxa*) of oil palm. The rhizopheric and endophytic isolates could fix N_2 under aerobic and microaerophilic conditions as determined by Acetylene Reduction Assay (ARA).

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