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Identification of Rhizobacteria from Ludwigia Octovalvis Grown in Arsenic

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Abstract

Rhizobacteria were isolated from the roots of Ludwigia octovalvis (Jacq.) Raven plants which grown in different places contaminated arsenic. Identification was conducted using two methods (Biolog GEN III and Vitek2 Compact) which were based on biochemical tests. All the identified rhizobacteria were Gram-positive bacteria: Arthrobacter globiformis, Bacillus megaterium, Bacillus cereus, Bacillus pumilus, Staphylococcus lentus, and also Gram-negative: Enterobacter asburiae, Sphingomonas paucimobilis, Pantoea spp, Rhizobium rhizogenes, Rhizobium radiobacter. The X code was identified as Rhizobium genus using Biolog GEN III method, this rhizobacteria was identified as R. radiobacter using Vitek2 Compact system. It indicates that two methods of identification could complement each other. Eleven rhizobacteria can be identified using Vitek2 Compact system, and another four rhizobacteria can be identified using Biolog GEN III method. In conclusion, Vitek2 Compact System method can identify more species of rhizobacteria than Biolog GEN III method.

Keywords: Arsenic, Root, Plant, Isolation, Rhizobacteria, Identification.

1. Introduction

Phytoremediation is a technology that uses green plants to remediate various media (soil, water or sediment) that are contaminated with different types of contaminants (organic and inorganic) and interact with microorganisms (ITRC, 2001; Ghosh and Singh, 2005; Cho-Ruk et al., 2006; Sao et al., 2007). Hyperaccumulating plant species, such as *Pityrogramma calomelanos* and *Pteris vittata*, were shown to accumulate arsenic in the form of arsenate at the leaf section (Visoottiviseth et al., 2002). The rhizosphere bacteria capability of aggressively colonizing plant roots and promoting plant growth are generally called the Plant Growth Promoting Rhizobacteria (PGPR) (Khan et al., 2009). PGPR such as *Agrobacterium* (*Rhizobium*), *Alcaligenes* (*Ralstonia*), *Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Serratia,* and *Pseudomonas* are particularly interesting for metal extraction by plants since they increase both the rates of metals accumulated by plants and the plant biomass (Lebeau et al., 2008).

Some research were conducted to evaluate the interactions of rhizobacteria and plant to remediate arsenic. According to Nie et al. (2002), the canola plants inoculated with *Enterobacter cloacae*, when grown in the presence of arsenic, grow to a signicantly greater extent than the non-transformed canola plants. Shilev et al. (2006) report that the shoot biomass and arsenic concentration in the shoot of *Heliathnus annuss* increase after being inoculated with *Pseudomonas fluorescens*. According to Titah et al. (2013a), the effect of applying the six-rhizobacterial consortium could alleviate the toxic effects of arsenic in *Ludwigia octovalvis* and increase the biomass weight of *L. octovalvis*.

L. octovalvis is one of the plants that can survive at a petroleum contaminated site (Rahman et al., 2009) and based on the conducted investigation, the plant could uptake and accumulates arsenic in their tissue (Titah et al., 2013b). The aim of the present study is to identify rhizobacteria that were isolated from roots of *L. octovalvis* after exposure to arsenic and roots of *L. octovalvis* grew at petroleum contaminated area in Malacca, Malaysia.

2. Material and Methods

2.1. Epiphyte Rhizobacterial Isolation from the Root of L. Octovalvis

The isolation of epiphyte rhizobacteria from *L. octovalvis* roots was carried out after 35 days of the arsenic exposure in greenhouse. Other isolation was conducted at petroleum contaminated site in Malacca in which *L. octovalvis* could grow. This isolation method is according to references of Abou-Shanab et al. (2005); Cakmakci et al. (2007), Harley and Prescott (2002), and Mittal and Johri (2009). Approximately 10 g of *L. octovalvis* roots with loosely attached soil from different concentrations of As-spiked sand was suspended in 100 mL sterile distilled water. It was shaken vigorously in an incubator shaker (Protech, Model SI-100D, Malaysia) at 37 °C and 150 rpm for 1 h. After all particles had been settled for 1 min, 1 mL of the homogeneous suspension was added to dilution tubes or a bottle containing 9 mL of sterile saline solution (8.5 g NaCl/1000 mL) to make a serial dilution (10^{-1} until 10^{-7}). The suspensions (0.1 mL) were plated onto a Trypticase (Tryptic) Soy Agar or TSA (Difco, USA) medium by a serial dilution using the spread plate technique. All plates were incubated at 37 ^{o}C in an incubator (Incucell, Germany), and were observed for 2 days.

2.2. Identification of Rhizobacteria

Rhizobacterial identification was conducted using two biochemical methods. First of all, the Biolog GEN III microbial identification system (Biolog Inc, USA) based on 71 carbon source utilization assays and 23 chemical sensitivity assays was used and the reading of identification results used the microStation semi-automated identification system (USA). Second, the rhizobacteria, which were not identified through Biolog GEN III, were later identified using Vitek2 Compact System (Biomerieux, USA) which was based on the differences of biochemical tests measuring carbon source utilization, inhibition and resistance, and enzymatic activities.

3. Results and Discussion

Based on previous study (Titah et al. 2011), 109 colonies were isolated which were further grouped into 29 groups of isolated rhizobacteria from roots of *L.octovalvis* after exposure to arsenic in

greenhouse. Meanwhile, there were 11 group of isolated rhizobacteria from roots of *L. octovalvis* at petroleum contaminated area. All the isolated rhizobacteria were given a code based on gram staining and morphological test on visual colony such as colour, shape, diameter, elevation, margin, and texture. The gram staining and morphological observation were conducted based on Harley and Prescott (2002). After that, all rhizobacteria isolated from roots of *L. octovalvis* were screened with arsenic to determine the arsenic tolerable rhizobacteria. Based on the arsenic screening results, twelve rhizobacteria isolated from the roots of *L. octovalvis* have the resistant to arsenic and were coded as B, C, G, J, K, M, Q, T, U, CC, Y and X, meanwhile another two rhizobacteria (LF-S1-3 and LF-S1-22) isolated from roots of *L. octovalvis* from a petroleum contaminated area have tolerance to arsenic. Number of rhizobacteria isolated at the greenhouse was higher than number of rhizobacteria isolated from contaminated site. It due to *L. octovalvis* grew at greenhouse was exposed only with arsenic. Meanwhile the petroleum contaminated area did not contain only arsenic but other heavy metals and organic pollutant. The main pollutant was organic pollutant as TPH (total petroleum hydrocarbons) with 144.5 ± 24.7 mg/kg, while heavy metals were arsenic (14.9 mg/kg), zinc (68.3 ± 2.4 mg/kg), chromium (2.2 mg kg-1), cuprum (4.5 ± 0.2 mg/kg), cadmium (0.3 mg/kg) and lead (9.8 ± 0.3 mg/kg) (Rahman et al. 2009).

Table 1 displays the results of the rhizobacterial identification. Based on the Biolog GEN III microbial identification system, the B isolate was identified as *A. globiformis* (probability 100%), the U code as *B. pumilus* (probability 100%), the CC code as *R. rhizogenes* (probability 88%) and the T code as *E. asburiae* (probability 96%). Maenwhile, the X code as *Rhizobium* genera. The rhizobacteria of C, G, J, K, M, Q, T, Y, LF-S1-3, and LF-S1-22 were not identified by Biolog GEN III.

Another method of Vitek2 Compact System was used to identify those unidentified rhizobacteria. The results show that the C code was identified as *Sphingomonas paucimobilis* (probability 86%), the G code was identified as *Sphingomonas paucimobilis* (probability 88%) and the J code was identified as *Bacillus cereus* (95%). The K code was identified as *B. megaterium* (probability 93%), the M code as *Staphylococcus lentus* (probability 88%), the Q code as *B. cereus* (probability 93%), the T code as *Enterobacter cloacae* (94%) and the Y code as *Bacillus pumilus* (93%). The LF-S1-3 code was identified as *Staphylococcus lentus* (probability 88%) and the LF-S1-22 as *Pantoea* spp (probability 98%). In addition, the X code was only identified as *Rhizobium* genus using Biolog GEN III method. However, using Vitek2 Compact system, this rhizobacteria was identified as *R. radiobacter* (probability 99%).

Based on the results of identification, the M code and the LF-S1-3 code were the same species rhizobacteria (*Staphylococcus lentus*) although the plant grew at two different places, greenhouse and contaminated area, respectively. The contaminated area where *L. octovalvis* grow was a petroleum contaminated area containing arsenic.

Results of rhizobacteria identification with T code using Biolog GEN III and Vitek2 Compact system showed different species. *E. asburiae* was identified using Biolog GEN III method but *E. cloacae complex* was identified using Vitek2 Compact system. According Paauw et al. (2008), *E. cloacae complex* is a complex bacteria, which consists of six species of bacteria namely *E. asburiae*, *E. cloacae*, *E. hormaechei*, *E. kobei*, *E. ludwigii* and *E. nimipressuralis*. Based on the result, rhizobacteria T was identified as *E. asburiae*.

Identification using Vitek2 Compact system resulted more identified rhizobacteria than Biolog GEN III method. This due to Vitec2 Compact system has higher database of bacteria than Biolog GEN III. Vitek2 Compact system has 2455 database of bacteria, with details 1436 database or bacillus, 562 database for Gram negative and 457 database for Gram positive (Anonymous, 2010a) . Meanwhile for Biolog GEN III method, it has 1405 database for bacteria, with details 1044 for aerobic bacteria species and 361 database for anaerobic bacteria species (Anonymous, 2010b).

Table-1. Summ ary of rhizobacterial identification

No	Rhizo-bacterial Code	Gram	Identification		Single Colony x40
			BIOLOG GEN III	VITEK2 COMPACT	
1	B	+	*Arthrobacter globiformis* (100%)	-	
2	C	-	No ID	*Sphingomonas paucimobilis* (86%)	
3	G	-	No ID	*Sphingomonas paucimobilis* (88%)	
4	J	+	No ID	*Bacillus cereus* (95%)	
5	K	+	No ID	*Bacillus megaterium* (93%)	
6	M	+	No ID	*Staphylococcus lentus* (88%)	
7	Q	+	No ID	*Bacillus cereus* (93%)	
8	T	-	*Enterobacter asburiae* (96%)	*Enterobacter cloacae* (94%)	
9	U	+	*Bacillus pumilus* (100%)	-	
10	X	-	*Rhizobium*	*Rhizobium radiobacter* (99%)	
11	Y	+	No ID	*Bacillus pumilus* (93%)	
12	CC	-	*Rhizobium rhizogenes* (88%)	-	
13	LF-S1-3	+	No ID	*Staphylococcus lentus* (88%)	
14	LF-S1-22	-	No ID	*Pantoea* spp (98%)	

- means it was not conducted

Many isolated rhizobacteria in this study were similar with the isolated rhizobacteria at other previous studies. *Bacillus* was a genus commonly isolated from various arsenic polluted area being identified in this study and other studies of Anderson and Cook (2004), Achour et al. (2007), Cavalca et al. (2010), Chopra et al. (2007), Jareonmit et al. (2012) and Valverde et al. (2011). Isolates *Arthrobacter* was identified in this study and a study was conducted by Achour et al. (2007) at arsenic contaminated site in France. *Enterobacter* as reported by Jareonmit et al. (2012) was also identified in this study. Srivastava et al. (2012) reported that *Staphylococcus sp* was isolated from rhizophera at contaminated area in Bengal, India. Macur et al. (2001) reported that *Sphingomonas* and *Rhizobium* were isolated at mining area. Yoon et al. (2009) reported that *Pantoea agglomerans* was isolated at arsenic contaminated area in South Korea.

4. Conclusions

Results based on Vitek2 Compact System method showed more species of identified rhizobacteria than Biolog GEN III method. Two methods of identification could complement each other. Eleven rhizobacteria can be identified using Vitek2 Compact system, meanwhile four rhizobacteria can be identified using Biolog GEN III method. Those rhiobacteria were identified as Gram-positive bacteria: *A. globiformis, B. megaterium, B. cereus, B. pumilus, Staphylococcus lentus* and Gram-negative: *E. asburiae, Sphingomonas paucimobilis, Pantoea* spp, *R. rhizogenes, and R. radiobacter*.

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