AENSI Journals

Australian Journal of Basic and Applied Sciences

ISSN:1991-8178

Journal home page: www.ajbasweb.com

Identification of Rhizobacteria from *Ludwigia octovalvis* Grown in Arsenic

[1,2,3,4]Harmin Sulistiyaning Titah, [1]Siti Rozaimah Sheikh Abdullah, [3]Idris Mushrifah, [1]Nurina Anuar, [2]Hassan Basri and [2]Muhammad Mukhlisin

[1]*Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.*
[2]*Department of Civil and Structural Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.*
[3]*Tasik Chini Research Centre, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.*
[4]*Department of Environmental Engineering, Faculty of Civil Engineering and Planning, Institut Teknologi Sepuluh Nopember (ITS), 60111, Keputih, Sukolilo, Surabaya, Indonesia.*

ARTICLE INFO

Article history:
Received 25 April 2014
Received in revised form
18 May 2014
Accepted 28 May 2014
Available online 2 June 2014

Keywords:
arsenic, root, plant, isolation, rhizobacteria, identification

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.

To Cite This Article: Harmin Sulistiyaning Titah, Siti Rozaimah Sheikh Abdullah, Idris Mushrifah, Nurina Anuar, Hassan Basri and Muhammad Mukhlisin., Identification of Rhizobacteria from Ludwigia octovalvis Grown in Arsenic. ***Aust. J. Basic & Appl. Sci.,*** *8(8): 134-139, 2014*

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.*

Corresponding Author: Harmin Sulistiyaning Titah, Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia.
Tel: +60149341284, E-mail: harmin_st@yahoo.co.id

octovalvis after exposure to arsenic and roots of *L. octovalvis* grew at petroleum contaminated area in Malacca, Malaysia.

MATERIAL AND METHODS

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.

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.

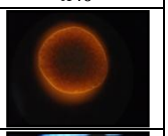
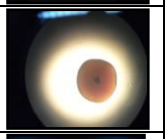
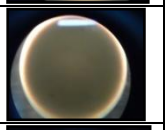
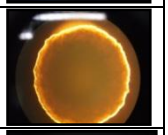
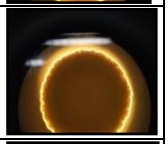
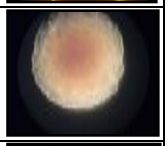
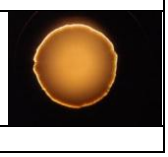
RESULTS AND DISCUSSION

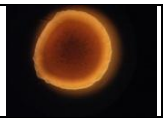
Based on previous study (Titah *et al*. 2011), 109 colonies were isolated which were further grouped into 29 groups of isolated rhizobacteria from roots of *L.octovalvis* after exposure to arsenic in greenhouse. Meanwhile, there were 11 group of isolated rhizobacteria from roots of *L. octovalvis* at 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%).

Table 1: Summary 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

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).

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.

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*.

ACKNOWLEDGEMENTS

The authors would like to thank Tasik Chini Research, Universiti Kebangsaan Malaysia (UKM) and the Ministry of Higher Education, Malaysia of ERGS/1/2013/TK05/UKM/02/2 for funding this research, and the Ministry of National Education of the Republic of Indonesia for providing doctoral scholarship to the first author.

REFERENCES

Abou-Shanab, R.A., H. Ghozlan, K. Ghanem and H. Moawad, 2005. Behavior of bacterial populations isolated from rhizosphere of *Diplachne fusc* dominant in industrial sites. World Journal of Microbiology and Biotechnology, 21: 1095-1101.

Achour, A.R., P. Bauda and P. Billard, 2007. Diversity of arsenite transporter from arsenic-resistant soil bacteria. Research Microbiology, 158: 128-137.

Anderson, C.R. and G.M. Cook, 2004. Isolation and characterization of arsenate-reducing bacteria from arsenic-contaminated sites in New Zealand. Current Microbiology, 48: 341-347.

Anonymous, 2010a. Vitek 2 Systems Product Information. bioMérieux, Inc. Durham, North Carolina, USA.

Anonymous, 2010b. GEN III Database Species and Their Characteristics. BIOLOG, Inc. Hayward, CA, USA.

Cakmakci, R., M.F. Donmez and U. Erdoan, 2007. The effect of plant growth promoting rhizobacteria on barley seedling growth, nutrient uptake, some soil properties, and bacterial counts. Turkey J. Agric. Forest, 3: 189-199.

Cavalca, L., R. Zanchi, A. Corsini, M. Colombo, C. Romagnoli, E. Canzi and V. Andreoni, 2010. Arsenic resistant bacteria associated with roots of the wild *Cirsium arvense* (L) plant from an arsenic polluted soil, and screening of the potential plant growth-promoting characteristics. Systematic and Applied Microbiolology, 33: 154-164.

Chopra, B.K., S. Bhat, I.P. Mikheenko, Z. Xu, Y. Yang, X. Lou, H. Chen, L. van Zwieten, R. McC. Lilley and R. Zhang, 2007. The characteristics of rhizophere microbes associated with plants in arsenic-contaminated soils from cattle dip sites. Science Total Environmental, 378: 331-342.

Cho-Ruk, K., J. Kurukote, P. Supprung and S. Vetayasuporn, 2006. Perennial plants in the phytoremediation of lead-contaminated soils. Biotechnology, 5(1), 1-4.

Ghosh, M. and S.P. Singh, 2005. A review on phytoremediation of heavy metals and utilization of its by product. Applied Ecology and Environmental Research, 3(1): 1-18.

Harley, J.P. and L.M. Prescott, 2002. Laboratory exercises in microbiology, Fifth Edition. McGraw−Hill Companies, Texas.

ITRC, 2001. Technical and regulatory guidance document, phytotechnology. Interstate Technology Regulatory Council USA. http://www.itrcweb.org/documents/Phyto-2.pdf.

Jareonmit, P., M. Mehta, M.J. Sadowsky and K. Sajjaphan, 2012. Phylogenetic and phenotypic analyses of arsenic-reducing bacteria isolated from an old tin mine area in Thailand. World Journal of Microbiology and Biotechnology, 28(5): 2287-2292.

Khan, M.S., A. Zaidi, P.A. Wani and M. Oves, 2009. Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils: A review. E. Lichtfouse (ed.), *Organic Farming, Pest Control and Remediation of Soil Pollutants, Sustainable Agriculture Reviews 1*, DOI 10.1007/978-1-4020-9654-9_5.

Lebeau, T., A. Braud and K. Jezequel, 2008. Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. Environmental Pollution, 153: 497-522.

Macur, R.E., J.T. Wheeler and T.R. Mcdermott, 2001. Microbial population associated with the reduction and enhanced mobilization of arsenic in mine tailings. Environmental Science Technology, 35: 3676-3682.

Mittal, S. and B.N. Johri, 2007. Assessment of rhizobacterial diversity of *Triticum aestivum* and *Eleusine coracana* from northern region of India. Current Science, 93(11): 1530-1537.

Nie, L., S. Shah, G.I. Burd, D.G. Dixon and B.R. Glick, 2002. Phytoremediation of arsenate contaminated soil by transgenic canola and the plant growth-promoting bacterium Enterobacter cloacae CAL2. Plant Physiology Biochemistry, 40: 355-361.

Paauw, A., M.P.M. Caspers, F.H.J. Schuren, M.A. Leverstein-van Hall, A. Deletoile, R.C. Montijn, J. Verhoef and A.C. Fluit, 2008. Genomic Diversity within the *Enterobacter cloacae* Complex. PLoS ONE, 3(8): 1-11.

Rahman, A., A. Amelia, A. Nazri, I. Mushrifah, N.S. Ahmad and A.A. Soffian, 2009. Screening of Plants Grown in Petrosludge - A Preliminary Study towards Toxicity Testing in Phytoremediation, Colloquium on UKM–PRSB Phytoremediation, Malaysia, August.

Sao, V., W. Nakbanpote and P. Triravetyan, 2007. Cadmium accumulation by *Axonopus compressus* (Sw.) P. Beauv dan *Cyperus rotundas* Linn growing in cadmium solution and cadmium-zinc contaminated soil. Journal of Science Technology, 29(3): 881-892.

Shilev, S., A. Fernandez, M. Benlloch and E.D. Sancho, 2006. Sunflower growth and tolerance to arsenic is increased by the rhizopheric bacteria *Pseudomonas fluorescen* , In : Morel, J.L, G. Echevarria, N. Goncharova (Eds.), Phytoremediation of Metal-Contaminated Soils, NATO Sciences Series, IV : Earth and Environmental Sciences, 68, 315-318.

Srivastava, S., P.C. Verma, A. Singh, M. Mishra, N. Singh, N. Sharma and N. Singh, 2012. Isolation and characterization of Staphylococcus sp. strain NBRIEAG-8 from arsenic contaminated site of West Bengal. Applied Microbiolology and Biotechnology, 95(5): 1275-1291.

Titah, H.S., S.R.S Abdullah, N. Anuar, M. Idris, H. Basri and M. Mukhlisin. 2011. Isolation and screening of arsenic resistant rhizobacteria of *Ludwigia octovalvis*. African Journal of Biotechnology, 10(81): 18695-18703.

Titah, H.S., S.R.S. Abdullah, I. Mushrifah, N. Anuar, N., H. Basri and M. Mukhlisin. 2013b. Arsenic toxicity on *Ludwigia octovalvis* in spiked sand. Bulletin of Environmental and Contamination Toxicology, 90(6): 714-719.

Titah, H.S., S.R.S. Abdullah, I. Mushrifah, N. Anuar, N., H. Basri and M. Mukhlisin. 2013a. Effect of applying rhizobacteria and fertilizer on the growth of *Ludwigia octovalvis* for arsenic uptake and accumulation in phytoremediation. Ecological Engineering, 58: 303-313

Valverde, A., M. Gonzalez-Tirante, M. Medina-Sierra and I. Santa-Regina, 2011. Diversity and community structure of culturable arsenic-resistant bacteria across a soil arsenic gradient at an abandoned tungsten-tin mining area. Chemosphere, 85: 129–134.

Visoottiviseth, P., K. Francesconi and W. Sridochan, 2002. The potential of Thai indigenous plant species for the phytoremediation of arsenic contaminated land. Environmental Pollution, 118: 453-461.

Yoon, I.H., J.S. Chang, J.H. Lee and K.W. Kim, 2009. Arsenite oxidation by *Alcaligenes* sp. strain RS-19 isolated from arsenic-contaminated mine in the Republic of Korea. Environmental Geochemistry and Health, 31(1): 109-117.