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The Potential of Bacteria Isolated from Earthworm Intestines, Vermicompost and Liquid Vermicompost to Produce Indole-3-Acetic Acid (IAA)

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Abstract Indole-3-acetic acid (IAA) is the most important member of the auxin class, which function as inducing cell division and cell elongation for plant growth and development. There are several Plant Growth-Promoting Rhizobacteria (PGPR), that colonize the rhizosphere and plant roots can producing IAA. But some groups of beneficial bacteria in nature do not move around; instead, they build colonies in one place. Because of this, they have little ability to promote the growth of plants in nature without being added into the soil at the root of the plants. This research we found that two IAA producing bacterial strains (A-2 and AnA-10) were isolated from the intestines of endogeic earthworms *M. posthuma* and identified as *Enterobacter* spp. based on 16S rDNA sequence homology were able to produced the amount of IAA 119.83 and 131.99 µg/ml. respectively. The results of this research both strains possess great potential to be developed as bio-fertilizers which might be enhance plant growth through IAA production with the presence of *M. posthuma* could led to multiply and divergent dynamics of bacterial community in soils.

Keywords: Earthworm, Vermicompost, Bacteria, Indole-3-acetic acid (IAA)

Introduction

The bacteria associated with earthworm intestines are most belong to the phylum Proteobacteria and the communities have been observed in three ecological groups of earthworms; epigeic, endogeic, and anecic earthworms (Thakuria *et al*., 2009). Earthworm intestines provide a unique anaerobic environment in the soil ecosystem and a mucus excreted by earthworm contribute to neutralizing soil pH, and then microbial activity increases. (Barois

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and Lavelle, 1986). Both the intestine microbes and the earthworm cooperate to the decomposition of nutrients in the soil, and they both benefit from this process, called a mutualistic digestive system (Drake and Horn, 2007).

Earlier research have been observed 343 types of microorganisms in the intestines of the earthworm *Pheretima* sp., including acitonomycetes, bacteria, and fungi (Winding *et al*., 1997). Most of these bacteria belong to the genus *Psudonomas* and *Azotobacter* (Valle *et al*., 2007; Byzov *et al*., 2007; Srikanjana *et al*., 2014), which can producing plant growth promoters, nitrogen-fixing bacteria, and phosphate solubilizers (Loreno *et al*., 2004; Martínez, 2001). In addition, Tancho, (2008) had reported that potted Gerbera plants in a mixture of soil and cow manure will yellow and die after one month. The electrical conductivity (EC) values can be measured at 2.1 dS/m. However, when potted with soil containing earthworms species *M. posthuma* , the trees grow normally, with an EC value decreased to 700 µS/cm. In addition, plots with earthworms have higher phosphorous (P) values, which is up to 40% more than plots without earthworms. This research demonstrates that earthworms play a role in improving the growth of plants, both in terms of nutrition and reducing the toxicity of unfermented manure. In addition, worm castings have a higher quantity of plant nutrition than soil with earthworms in it, due to the microorganisms in the worm castings, the greater amount of bacteria in the worm castings, and the activity of enzymes, acid and alkaline phosphates which is greater than what can be measured from the earthworm feed (Manuel and Jorge, 2009).

Therefore, the objective of this research was to isolate and screen indigenous Indole-3- acetic acid producing bacteria from different species of earthworm intestines, including endogeic earthworms *M. posthuma* collected from agriculture land, epigeic earthworms *Perionyx* sp. collected from dairy farm and vermiculture and their product vermicompost and liquid vermicompost. Isolated bacteria could have the potential as PGPR.

Materials and methods

Isolation of IAA producing bacteria from earthworms vermicompost and liquid vermicompost

This study utilized earthworms from different environments:- *Metaphire posthuma* the most abundant species were taken from a farm plot at Maejo University the epigean *Perionyx* sp. were taken from dairy farms in Amphur Sankampaeng, Chiang Mai Province while more *Perionyx* sp. earthworms, as well as their castings and liquid vermicompost, were collected from a commercial vermicomposting farm. Twenty mature earthworms of each kind

were selected and their intestines cut open at the section known as the 'hindgut'. They were then ground until fine using sterilized equipment.

Serial dilutions of five bacterial isolates were carried out on mixed samples containing approximately 1 gram of intestines, vermicompost and liquid vermicompost. Prepared in a 0.85% saline solution 0.1 ml aliquots of 10^6 dilution were then plated onto nutrient agar (NA) and de Man, Rogose, and Sharpe agar (MRS agar). After incubation at 30 ˚C in both aerobic and anaerobic conditions for 24 hours, single bacterial colonies from triplicate plates were identified. Single colonies were picked and streaked again onto nutrient agar plates to obtain pure cultures. Stock cultures were made in nutrient broth containing 20% (w/v) glycerol and stored at -86 ◦C.

Screening of isolated for Indole-3-acetic acid (IAA) Production

Quantification of the IAA produced by the 68 strains of bacteria isolated from the intestines of earthworms, vermicompost and liquid vermicompost was done by growing the bacterial strains in a minimal salt (MS) medium amended with 5 mM L-Tryptophan (Frankenberger and Poth, 1988). The concentration of IAA produced was then determined using the colorimetric method (Gordon and Weber; 1951). IAA production was qualitatively analyzed by growing 50 µl of bacterial strains on 5 ml of nutrient broth (NB) containing 5 g NaCl, 10 g Peptone and 10 g beef extract) incubated at 30 ºC for 24 hours. A subculture of 100 µl was then grown on a minimal salt (MS) medium containing 1.36 g/l KH_2PO_4, 2.13 g/l Na_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$ and other trace elements amended with 5 mM L-Tryptophan (a stock solution of L-Tryptophan was used containing 10 g Glucose, 1 g L-Tryptophan, and 0.1 g yeast extract sterilized using filter paper of 0.2 µm diameter). The solution was incubated at 30 ºC for 36 hours before determining the IAA produced using the colorimetric method. The supernatant was harvested by centrifuge at a rate of 12,000 rpm for 5 min at 4 ºC and then mixed with $FeCl_3$-$HClO_4$ at a ratio of 1 ml: 1 ml and incubated at room temperature for 25 minutes. Absorbance was measured at 530 nm calculated from a single equation of standard curve derived from the concentration of IAA produced from each bacterial strain.

Identification of isolates

DNA was extracted using a Gel/PCR DNA Fragments Extraction Kit (Geneaid®). The 16S rRNA genes were then amplified using the Polymerase Chain Reaction (PCR) method with primers 20F (5' GAG TTT GAT CCT GGC TCA G 3') and 1500R (5' GTT ACC TTG TTA CGA CTT3'). The

amplification product was then verified on 0.8% agarose gel, purified using a Quick PCR purification column (Sigma, WI, USA) and sequenced with an automated DNA sequencer using the primer 800R (5' TAC CAG GGT ATC TAA TCC 3') and the facility at Macrogen Inc. (Seoul, Korea). Molecular phylogenetic analyses were done using the software MEGA 5.0, by comparing the test sequence with reference sequences downloaded from the eztaxon-e database available on the site http://eztaxon-e.ezbiocloud.net/ezt_identify. Evolutionary correlations were then analysed and compared with other type strains by making a phylogenetic tree using the programmes BioEdit and MEGA 6.

Statistical analysis

The data were subjected to analysis of variance (ANOVA), and Duncan's Multiple Range Test (DMRT) at $p \leq 0.05$ was used to determine treatment mean differences.

Results

Isolation of earthworm intestines, vermicompost and liquid vermicompost bacterial strains and screening for IAA production

Sixty-eight isolates of earthworm intestines, vermicompost and liquid vermicompost bacterial were isolated and screened for the production of IAA. Results revealed that all 68 bacterial strains can producing Indole-3-acetic acid ranging from 0.33 to 131.00 µg/ml. The largest group of bacteria consisted of 61 strains (89%) showed low production ability of IAA (<1-32 µg/ml), 5 strains (7.35%) showed moderate production ability of IAA (51-80 µg/ml), and 2 strains (2.94%) showed high production ability of IAA (119-131 µg/ml). (Table 1) In comparison, 8 bacterial strains, which produce IAA in high concentrations between 32-131 µg/ml. It was found that IAA production by the bacteria isolated from the intestines endogeic earthworms *M. posthuma* was higher than the bacteria isolated from the intestines of epigean earthworms *Perionyx* sp. significantly ($p \leq 0.01$) (Table 1 and Table 2) .

Table 1. Screening of IAA-producing by bacteria isolated from the intestines of earthworms, vermicompost and liquid vermicompost including 68 isolates

Isolates code	IAA level (µg/ml)	Isolates code	IAA level (µg/ml)	Isolates code	IAA level (µg/ml)
A-2	**131.39**	P1-16	6.40	V-20	0.45
AnA-10	**119.83**	P1-15	6.20	V-10	0.43
A-5	**71.39**	P1-8	4.55	V-9	0.43
AnA-3	15.28	P1-5	3.59	V-7	0.39
AnA-9	3.55	P1-20	2.82	V-12	0.34
A-10	1.84	P1-13	0.84	LV-18	5.10
A-7	0.60	P1-12	0.76	LV-4	3.55
A-6	0.46	P1-10	0.57	MRSLV-	3.24
AnA-4	0.33	P1-1	0.55	LV-3	2.05
AnPo-3	**72.80**	P1-2	0.55	MRSLV-	1.95
Po-10	**65.28**	V-22	6.27	MRSLV-	1.48
Po-9	**51.74**	V-1	4.68	MRSLV-	1.48
Po-4	**32.45**	V-18	1.76	MRSLV-	1.18
Po-12	12.97	V-17	1.24	MRSLV-	0.78
Po-14	7.69	V-23	1.16	LV-14	0.70
Po-15	5.41	V-3	0.97	LV-15	0.65
Po-2	0.82	V-15	0.94	LV-7	0.56
Po-6	0.65	V-5	0.76	LV-6	0.53
Po-5	0.51	V-2	0.75	LV-13	0.51
Po-13	0.44	V-6	0.73	LV-9	0.45
AnP1-6	**80.44**	V-24	0.64	MRSLV-	0.38
P1-11	8.15	V-11	0.59	LV-2	0.33
P1-19	6.65	V-4	0.53		

Table 2. The bacteria consisted of 8 isolates which produced IAA in high concentration levels

Sampling site	Sampling	Isolates code	IAA (µg/ml)
A farm plot at Maejo University, Chiang Mai	Intestines of endogeic earthworms *M. posthuma*	A-2	131.39 ± 1.74^{A}
		A-5	71.39 ± 0.69^{CD}
		AnA-10	119.83 ± 2.66^{B}
Dairy farms in Amphur Sankampaeng, Chiang Mai	Intestines of epigean earthworms *Perionyx* sp.	Po-4	32.45 ± 0.23^{F}
		Po-9	51.74 ± 3.08^{E}
		Po-10	65.28 ± 1.88^{D}
		AnPo-3	72.80 ± 1.46^{CD}
A commercial vermicomposting farm, Chiang Mai	Intestines of epigean earthworms *Perionyx* sp.	AnP1-6	80.44 ± 3.58^{C}
% CV			4.84
F-test			**

**Mean with the different letters are significantly different at $p \leq 0.01$ by the Duncan's Multiple Range Test , Values given here are the means (n=3) ± standard deviation.

Identification of the bacterial strain

According to 16S rRNA sequence homology and subsequent molecular phylogeny analysis. The 2 bacterial strains A-2 and AnA-10 which were isolated from the intestines of earthworm species *Metaphire posthuma* collected from agricultural land, indicated highest IAA production showed closest match with the genus *Enterobacter*. The 4 bacterial strains AnP1-6, AnPO-3, Po-10, Po-9 and which were isolated from the intestines of earthworm species *Perionyx* sp. collected from a commercial vermicomposting farm and dairy farm closest match with the genus *Aeromonas*, while the bacterial strain A-5 and Po-4 showed close proximity with the genus *Bacillus* and *Aeromonas* respectively (Table 3 and Figure 1).

Table 3. The classification of bacteria 8 isolates which produced IAA in high concentration levels

Isolates code	Identify as			
	Length (bp)	Closest match	% Similarity	Accession number
A-2	622	*Enterobacter hormaechei* ATCC 49162^T	99.36	AFHR01000079
AnA-10	692	*Enterobacter cloacae* subsp. *dissolvens* LMG 2683^T	99.57	Z96079
AnP1-6	692	*Aeromonas punctata* subsp. *punctata* NCIMB 13016^T	100.00	X60408
AnPo-3	672	*Aeromonas sanarellii* A2-67^T	99.70	FJ230076
A-5	662	*Aeromonas sanarellii* A2-67^T	99.55	FJ230076
Po-10	690	*Aeromonas enteropelogenes* ATCC 49657^T	99.27	X60415
Po-9	690	*Aeromonas media* ATCC 33907^T	99.27	X74679
Po-4	670	*Bacillus aryabhattai* B8W22^T	100.00	EF114313

Discussion

Earlier research had shown that over 26% of bacteria isolated from vermicompost which were able to produce IAA belonged to the groups *Pseudomonas*, *Bacillus*, *Arthrobacter*, and *Enterobacter* (Madhuri and Sahasrabudhe, 2011; Wahyudi *et al*., 2011). In the present study, isolated 68 earthworm intestines, vermicompost and liquid vermicompost bacterial and they were screened for IAA production. Based on the results of IAA production, two *Enterobacter* strains, five *Aeromonas* strains and one *Bacillus* strains with outstanding performances were selected for compared with earlier research, this study was able to isolated bacteria that were able to produce IAA at higher levels than the research of Jayakumar (Jayakumar and Natarajan, 2013) which isolated 51 isolates of bacteria from vermicompost made from the castings of earthworms *Eisenia foetida*, which were able to produce IAA at levels of 1.50 to 36.00 µg/ml. Bacteria of the strain *B. subtilis* BVC53 produced the highest levels of IAA at 36.00 µg/ml (Khare and Arora, 2010). And 5 strains of bacteria isolated from the soil in longan orchard were able to produced IAA at the level 11.4-57.6 µg/ml (Srikanjana *et al*., 2014). In addition, bacteria from the strain *Pseudomonas* sp. TLC 6-6.5-2 produced the highest IAA at 7.16 µg/ml (Kefeng and Wusirika, 2011) and Charin (2011) research demonstrated that *Azospirillum* isolate S^{23} was able to produce IAA at

the highest levels of 53.57, 31.85 and 22.57 µg/ml at the first, second and third days after incubation, respectively (Charin, 2011). However according to Walpola and Arunakumara (2015) they recorded the maximum IAA production 240 and 332 µg/ml respectively in *E. ludwigii* and *E. Hormaechei* phosphate solubilizing and indole acetic acid (IAA) producing bacteria isolated from green house soils.

The two strains of *Enterobacter* founded in this work that are the strains with the capability to produce the highest values of IAA, could be beneficial for the growth of plants, however IAA observed under laboratory conditions could be different to that produced associated to earthworms or plants. For example, gene expression depends of culture media were bacteria is growing, the environment and the host (Ramos-Gonzalez *et al*., 2005; Wasarman, 2002; Ona *et al*., 2005). Bacteria of genera *Enterobacte*r has been published as PGPR both under greenhouse conditions, such as under field conditions (Ogbo, 2012; Kryuchkova *et al*., 2014; Morales-García *et al*., 2011). Therefore, bacteria isolated at the present work could be also PGPRs when they are associated to plants.

Conclusions

This study we found that almost 8 isolated bacteria that have a high capable to produce high IAA were belonged to the intestines of both species of earthworm, *M. posthuma* and *Perionyx* sp. Bacterial strains A-2, AnA-10 isolated from intestines of an endogeic earthworm *M. posthuma* collected from agricultural land identified as genera *Enterobacter*, *Aeromonas* were able to produce the highest amount of IAA 119.83-131.99 µg/ml. We already have deposit Bacterial strains A-2, AnA-10 for public access at Thailand Bioresource Research Center (TBRC), so we plan to extend research to be used in order to make them more effective for agricultural purposes soon to management farming systems especially organic farming. To promote plant growth and development.

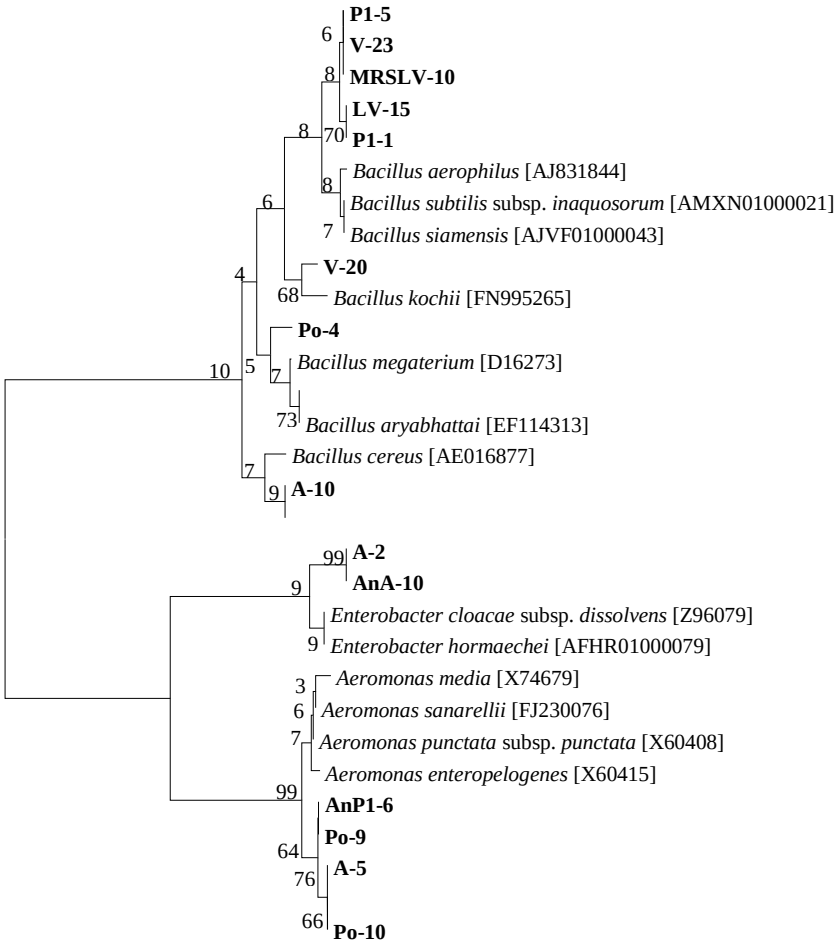

Figure 1. Phylogenetic analyses of producing highest amount of Indole--3acetic acid (IAA) bacteria which isolated from intestine of earthworm, vermicompost and liquid vermicompost based on the nucleotide sequence of 16S rRNA.The phylogenetic tree was constructed by neighbour-joining (NJ) method with 10,000 replicates using bootstrap.

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