Isolation and Characterization of Multifunction Beneficial Bacteria From Dairy Farm Effluent Compost

Noor Khairani Mohamad Basri[1], Januarius Gobilik[1], Lum Mok Sam[1], Chong Khim Phin[2], Kimberly Ador[1], Mohamadu Boyie Jalloh[1], Clament Chin Fui Seung[1#]

1 Faculty of Sustainable Agriculture, Universiti Malaysia Sabah, Sandakan Campus, Mile 10, Sg. Batang, 90000, Sandakan, Sabah, MALAYSIA.
2 Faculty of Science and Natural Resources, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, MALAYSIA.
#Corresponding author. E-Mail: clament@ums.edu.my; Tel: +6088-320000; Fax: +6088-435324

ABSTRACT Compost is a good biomass reservoir of a broad range of microbial communities, with bacteria, fungi, and actinomycetes being the main microorganisms groups reported in the literature. Some bacteria assigned as plant growth-promoting agents are capable of enhancing plant growth and improving soil fertility. In this study, bacteria with multiple beneficial traits for potential use in agriculture were screened and characterized from the dairy farm effluent (DFE) compost. A total of 160 bacterial colonies originally picked from 11 selective media were purified and used in this study. The variations among the isolates in plant growth beneficial traits were studied by agar plate and spectrophotometric assays. Results revealed 38 isolates exhibited multiple plant growth beneficial traits and there were nitrogen fixation, solubilization of potassium, zinc silicate, and organic and inorganic phosphorus, as well as production of iron-chelating siderophore, chitinase, protease, 1-aminocyclopropane-1-carboxylate (ACC)-deaminase, indole-3-acetic acid (IAA), and hydrogen cyanide (HCN). From the 160 isolates, 34.38% were label as high IAA production isolates, 33.75% were able to metabolize ACC, 8.13% were able to produce high HCN, and the remaining 23.82% isolates showed moderately low or absence of activities. These data suggested that DFE compost contains multifunction beneficial traits bacteria and its utilization on agricultural crops has the potentials to influence plant health and productivity in various ways. These include fixation of nitrogen, enhance mobilizing of insoluble soil minerals such as P and K, as well as provide basal protection against plant pathogens through HCN production and chitinase activity. As a summary, this study showed DFE compost is a potent plant growth booster with multiple effective microorganisms and has the potential application in novel bio-fertilizer formulations for the Malaysian agriculture sector.

KEYWORDS: Dairy farm effluent, IAA, ACC-deaminase, Chitinase, HCN, Siderophore
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INTRODUCTION

Dairy farms generate a considerable amount of waste like manure and effluent. When undergoing proper composting stages, it can apply in cropland as an organic fertilizer amendment. While the use of dairy farm waste as a soil amendment can generally improve the soil structure, it is increasingly regarded as resource use for its minerals content rather than disposal waste. Besides, compost also functions as a reservoir for microorganisms. It contains an abundance of bacteria, some of which are beneficial bacteria towards plant growth, known as the plant growth-promoting bacteria (PGPB). PGPB of compost origin and has the potential application in agriculture practices were isolated and reported from various composts, including oil palm-based EFB-POME compost, kitchen refuses compost, and marine animal resource compost (Chin *et al.*, 2017; Kitpreechavanich *et al.*, 2016). This study was conducted to evaluate beneficial bacteria with plant growth-promoting traits present in the DFE compost.

PGPB promotes plant growth directly and indirectly (Glick, 1995). Direct mechanisms include the ability to solubilize the insoluble forms of phosphorus (P), and potassium (K) that are fixed in the soil matrix, fixing atmospheric nitrogen (N), and produce plant growth-regulating hormones such as

indole-3-acetic acid (IAA). The indirect mechanisms involve antagonisms against phytopathogenic microorganisms via secreting antimicrobial metabolites like iron-chelating siderophores, cyanide, and hydrolytic enzymes such as chitinase and proteases. Also, PGPB alleviates the various stress by secreting ACC (1-aminocyclopropane-1- carboxylate) deaminase enzyme and control disease by suppressing or killing the phytopathogens (Dell'Amico *et al.*, 2005).

Previously, we reported the direct effect of DFE compost on the enhancement of growth of Pak Choy (*Brassica rapa L.*) in the Pot System (Maludin *et al.*, 2019). However, research on screening beneficial bacteria from the DFE compost has been very scanty. Early research on DFE compost was more on the physicochemical properties and nutrient contents. This study aimed to isolate bacteria with multiple beneficial traits that could be used in the future to improve plant growth, increase soil fertility, and suppress phytopathogens. The main objective of this study, therefore, is to screen potential cultivable bacteria with multiple beneficial traits present in the DFE compost.

METHODOLOGY

Isolation of Bacteria.

10 g of DFE compost were transferred to 90 ml sterile distilled water and mixed thoroughly by shaking the flask on a rotatory shaker for 60 min. one ml of the diluent was serially diluted until 104, and 0.1 ml suspension was spread through on each of the following media: Mueller Hinton Agar, modified oil palm leaf agar, and oil palm root agar (dextrose 1g, dipotassium phosphate 7g, monopotassium phosphate 2g, sodium citrate 0.5g, magnesium sulfate 0.1g, ammonium sulfate 1g, agar 15g, and 20% aqueous oil palm leaf extract or 20% aqueous oil palm root extract, respectively, pH 7.0±0.20), Pikovskaya agar, modified CIRP (Christmas Island Rock Phosphate) agar, actinomyces agar, nitrogen-free agar, chitin agar, carboxymethyl cellulose agar, Luria-Bertani agar, and Tris minimal agar. All inoculated plates were incubated at 30 ± 2°C for 48-240 h. The colonies which showed variation in morphology were picked and purified for further screenings.

Mineral Solubilization

Screening of P-solubilization ability of the isolates was carried out using Pikovskaya's (PVK) agar plates (Pikovskaya, 1948) and National Botanical Research Institute's Phosphate Solubilization (NBRIP) media supplemented with sources of phosphorus; Ca_3 $(PO_4)_2$ and Fe_3PO_4, respectively. K-solubilization ability of isolates was screened using Aleksandrov agar plates which contain Mica powder ($KAlSi_3O_8$) as a source of insoluble inorganic potassium. Zinc (Zn) solubilization ability of isolates was screened using Tris-minimal medium plates contain zinc phosphate and zinc carbonate as a source of insoluble inorganic Zn. Meanwhile, the N-fixing ability of the isolates was tested on N-free medium agar. Inoculated plates were then incubated at 30 ± 2°C for 5-7 days and observed for growth and the clearing zone around the colonies.

Enzyme Activity

Phytase activity of individual isolates was screened using NBRIP media supplemented with soy lecithin as a sole source of organic phosphate. Protease activity was screened using skim milk agar assay. Chitinase production was screened using minimum medium agar amended with chitin as the sole source of carbon (Dunne *et al.*, 1997). The qualitative assay for chitinase and incubated for seven days. These plates were incubated at 30 ± 2°C for 3 days and examined for the development of clear zones around colonies. On the other hand, the production of ACC deaminase was determined as described by Dell'Amico *et al.* (2005). Briefly, ACC-deaminase producing bacterial isolates use amino

cyclopropane-1 carboxylic acid (ACC) as N source. After 48 h, light absorbance was measured at 530 nm using a microplate reader and data were presented as ACC deaminase production index.

Indole-3-Acetic Acid (IAA), Siderophore, and HCN Production

The production of indole-3-acetic acid (IAA) by bacterial isolates was determined using the Gordon and Weber methods with orthophosphoric acid and Salkowski reagent (Etesami *et al.*, 2014). Siderophore production was determined on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (Adriane *et al.*, 1999). HCN (cyanogen) production was screened using a modified method described by Shahla *et al.* (2016). The cultures were incubated at 30 ± 2°C for 24h, 3 days, and 5 days for the detection of IAA, siderophore, and HCN production, respectively. The pink appearance of the supernatant indicated a positive on IAA production. The formation of orange to yellow halo around the colonies indicated the production of siderophore. Development of color from yellow to brown on Whatman filter paper pre-soaked in sodium picrate solution on the top of the Petri dish plate was examined for putative HCN production.

RESULT AND DISCUSSION

Microorganisms including bacteria and fungi are commonly inhabiting the compost. In this study, 160 culturable bacteria isolates were selectively picked and purified from the DFE compost. The number of bacteria isolated from each media were designated as follow: modified oil palm root agar (RA, 28 isolates), modified oil palm leaf agar (LA, 20 isolates), Mueller Hinton agar (MH, 19 isolates), Pikovskaya agar (PKV, 16 isolates), Actinomyces agar (AIA, 15 isolates), Luria-Bertani agar (LB, 11 isolates), modified CIRP agar (13 isolates), N-free agar (12 isolates), CMC agar (10 isolates), chitin agar (9 isolates), and tris minimal agar (7 isolates). These results showed DFE compost is a reservoir of a broad range of bacterial communities with various potent activities relates to agriculture.

Phosphorus, Potassium, and Zinc Solubilization

In the natural environment, microorganisms facilitate the solubilization of complex compounds before the absorption of essential nutrients such as phosphorus. A total of 62 bacteria isolates (38.75%) from DFE compost were found to possess the ability of solubilizing inorganic P with clear zones ranged between 1.0 cm to 3.9 cm diameters around colonies in the PVK agar (Figure 1a). These bacteria isolates were further tested for phytase production where only 32.5% were capable of solubilized organic-P. Among 160 bacteria isolates tested only 41 isolates (25.63%) were able to solubilize K (Figure 1b). Meanwhile, 51 (31.88%) isolates of bacteria were able to solubilize zinc silicates (Figure 1c) and 57 (36.63%) isolates were classified as N-fixing bacteria (Figure 1d).

The development of plants required optimum amounts of essential elements N, P, and K for healthy growth. The ability of isolates to grow on N-free media indirectly indicates nitrogenase activity and the capability of the N-fixing isolates to fix N directly from the atmosphere (Parmar & Dadarwal, 1999). Rodriguez & Fraga (1999) and Parmar & Sindhu (2013) reported that P-solubilizing and K-solubilizing bacteria could enhance plant growth and yield by releasing phosphorus and potassium from P-bearing and K-bearing minerals in soils through the accessible form, making them available for plant absorption. Besides, K-solubilizing bacteria found to dissolve potassium, silicon, and aluminum from insoluble K-bearing minerals such as micas, illite, and orthoclases by excreting organic acids or chelating silicon ions to bring K into the solution. On the other hand, the ability to grow on zinc silicate-containing media indicates some isolates could tolerate the heavy metal Zn and solubilize Si minerals to orthosilicic acid; a plant-absorbing form. In addition, the ability to solubilize silicate could also pave the way to release other essential elements from soil silicate minerals. These

include P, K, Fe, Ca, Mg, and Na (Vasanthi *et al.*, 2013). Hence, DFE compost is an organic amendment that could enhance the mobilization of insoluble organic and inorganic P, mineral K, and silicate, both in the compost and soil, towards healthier plant growth via microbial activities.

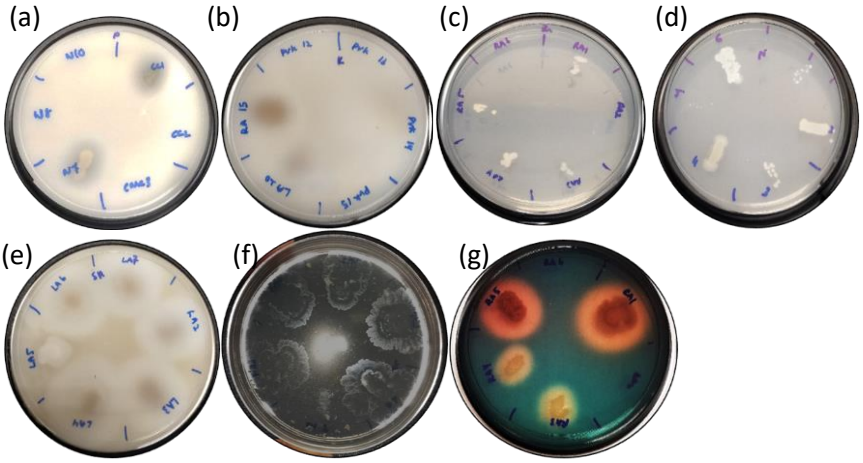

Figure 1. Representative images of *in vitro* screening on DFE compost isolates. (a) P-solubilisation indicated by clear zone on PVK agar, (b) K-solubilisation indicated by clear zone on Alekxandrov agar, (c) bacteria growth on minimal zinc silicate agar indicate silicate solubilisation, (d) bacteria growth on N-free agar indicate ability in fixing atmospheric N_2, (e) protease production indicated by clear zone on skim milk agar, (f) chitinase production indicated by halo zone on chitin agar, and (g) siderophore production isolates indicated by iron-rust colour around the colonies (bar = 3 cm).

Protease, Chitinase, Siderophore, Hydrogen Cyanide, ACC Deaminase, and IAA Production

The bacteria isolates were screened further to examine their biological control traits. The results, 94 (58.75%) isolates were able to produce protease enzyme on skim milk agar (Figure 1e), 65 (40.63%) isolates were chitinase producers (Figure 1f), 64 (40.0%) isolates were siderophore producers (Figure 1g), 55 (34.38%) isolates were IAA producer, 13 (8.13%) isolates were hydrogen cyanide producers, 105 (65.63%) isolates were able to produce ACC deaminase (range from 0.201-1.00 µg/ml), and 54 (33.75%) isolates were classified as a good ACC deaminase producer which produces more than 0.901 µg/ml. Overall, a total of 38 (23.75%) out of 160 isolates in this study were categorized as multifunction beneficial bacteria with both PGP properties and biocontrol traits (Table 1).

Chitinase, siderophore, and HCN production bacteria are potential biological control agents toward soilborne disease pathogens and insect pests for healthy plant growth. Chitinase-producing bacteria could exhibit antagonism behaviors by enzymatically degrading the chitin-containing cell walls of fungi and insects (Herrera-Estrella & 1999). Next, siderophore is a low molecular chelator that has a high affinity towards ion Fe^{3+} (Adriane *et al.*, 1999). During the colonization stage, siderophore-producing bacteria could compete with phytopathogens at the rhizosphere for iron uptake. In the late stage, the scavenged irons could be available to plant roots after lysis of the bacterial cells. In other words, siderophores-producing bacteria are an iron carrier, which also acts as potential biocontrol agents (Hu & Xu 2011). Meanwhile, HCN is a potent inhibitor of cytochrome-C oxidase and of several other metalloenzymes that are involving in electron transport chain during microbial respiration (Nuskova *et al.*, 2010). Chitinase, siderophore, and HCN producing bacteria can thus, help plants in their defense against phytopathogens.

TRANSACTIONS ON SCIENCE AND TECHNOLOGY

Ethylene is a gaseous plant hormone that regulates growth and development. However, excessive ethylene production may occur due to biotic and abiotic stresses, sending false regulation signals to the plant cells and cause harm to the plant. A prominent mechanism used by many PGPB is to facilitate plant growth by reducing the level of amino cyclopropane-1 carboxylic acid (ACC), the immediate precursor of ethylene, through the regulation of ACC deaminase (ACCD) synthesis (Egamberdiyeva & Hoflich, 2004). Thus, isolates that exhibiting ACCD activity can partially prevent any reduction in the length of plant roots, shoots, and biomass caused by high ethylene levels (Glick, 1995, Parmar & Dadarwal, 1999). Another plant hormone the indole-3-acetic acid (IAA) or auxin, is also known to be produced by microorganisms. It is known to stimulate cell elongation, cell division, and differentiation in plants (Chaiharn & Lumyong, 2011). Nevertheless, this hormone is also showed to participating in defense-related reactions and inhibit fungal mycelium growth and spore germination (Yu *et al*., 2008).

Table 1. Semi quantitative screenings of beneficial traits of selected bacterial isolates

Isolates	Plant Growth Promoting (solubilization)					Biocontrol (production)					
	N (fixation)	P (inorganic)	P (organic)	K	Zn Silicate	Protease	Chitinase	ACC deaminase	IAA	Siderophore	**HCN
MH5	+	+	-	-	+	+	+	0.140	0.278	+	+
MH6	+	+	-	+	-	-	+	0.172	0.260	+	+
MH8	-	+	-	+	-	+	+	0.143	0.308	+	+
MH12	+	+	-	+	+	+	+	0.413	0.232	+	+
MH13	+	+	-	+	+	+	+	0.211	0.223	+	+
MH18	+	+	-	-	+	+	+	0.349	0.248	+	+
LA1	-	+	-	-	+	+	+	0.197	0.345	+	+
LA2	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.160**	**0.364**	**+**	**+++**
LA3	+	+	+	+	+	+	+	0.164	0.344	+	++
LA4	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.144**	**0.419**	**+**	**+++**
LA6	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.194**	**0.388**	**+**	**+++**
LA7	+	+	+	+	+	+	+	0.183	0.377	+	++
LA8	+	+	-	-	+	+	+	0.320	0.348	+	+
LA11	+	+	+	-	-	-	-	0.459	0.247	+	+
LA18	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.363**	**0.323**	**+**	**+++**
LA19	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.493**	**0.275**	**+**	**+++**
LA20	+	+	+	-	+	-	-	**0.949**	0.131	+	+
RA1	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.220**	**0.241**	**+**	**+++**
RA3	-	-	+	+	-	+	+	0.423	0.233	+	+
RA4	+	-	+	+	-	+	+	0.504	0.233	+	+
RA5	+	+	+	+	+	+	+	0.183	0.256	+	++
RA8	+	+	+	+	+	+	+	0.113	0.244	+	++
RA16	+	+	+	+	+	+	-	0.400	0.194	+	+
RA17	-	+	+	-	+	+	+	0.250	0.239	+	+
RA20	+	+	+	-	-	-	+	0.467	0.282	+	+
RA23	+	+	+	+	+	-	-	0.485	0.197	+	+
RA24	+	+	+	-	+	+	-	0.182	0.210	+	+
RA27	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.202**	**0.125**	**+**	**+++**
PVK1	-	+	-	-	+	+	+	0.208	0.308	+	+
PVK5	+	+	+	-	+	+	+	**0.912**	0.363	-	+
CIRP2	+	+	+	+	-	-	+	0.362	0.190	-	+
AIA1	+	+	+	+	+	+	+	0.171	0.231	+	++
AIA6	+	+	+	+	+	+	+	0.298	0.163	+	++
AIA15	**+**	**+**	**+**	**+**	**+**	**+**	**+**	**0.948**	**0.297**	**+**	**+++**
N1	+	-	-	-	+	+	-	0.434	**0.445**	+	+
N3	+	+	+	+	+	+	+	0.671	**0.487**	+	++
N7	+	+	-	+	+	+	-	0.300	0.399	+	+
CC1	+	+	+	+	+	+	+	0.182	0.110	+	++

Note: +, detected; -, not detected. All measurement were done on triplicates.

** The colour of filter paper in the HCN test for cream (+), light brown (++), and dark brown (+++)

CONCLUSION

Soil is rich in minerals but not all elements are accessible by plant and readily available for root uptake due to the complex forms present in the soil matrix. Bacteria exhibit multifunctional traits that inhabit the DFE compost have the potential to aid in the breaking down of the soil matrix complex. The current study on the characterization of DFE compost inhabiting bacterial isolates indicates DFE compost has high potency use in crop production practices. Results suggested when applying as an organic soil amendment biomass, DFE compost has the potential to enhance plant growth via mineralization of organic and inorganic elements of P, K, Zn, and silicate-based elements. When using as a planting medium, it shows the potential to provide basal protection against soilborne pathogens simultaneously reduce disease incident via the secretion of siderophore, chitinase, IAA, and HCN. Several bacterial isolates (as highlighted in Table 1) also exhibited the potential to be used as biofertilizer and biocontrol agents. A future study using selected bacterial isolates on plant growth is necessary to evaluate their plant growth-promoting performance in the laboratory scales and the field study. Last but not least, some isolates could be exploited for enzyme production in the future, such as chitinases, cellulases, and proteases that know to have a very high commercial value.

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