Isolation and characterization of plant growth promoting bacteria (PGPB) from anaerobic digestate and their effect on common wheat (*Triticum aestivum*) seedling growth

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Abstract— *The use of anaerobic digestate as fertilizer is considered beneficial since it provides plant nutrients and organic matter to soils. However, there is limited information about plant growth promoting bacteria (PGPB) in digestate. In this study, we isolated Bacillus and Pseudomonas from two types of anaerobic digestates, and selected three different plant growth promoting traits and antifungal activity to screen 200 bacteria isolated from each digestate. Then 6 isolates based on plant growth promoting traits were selected and inoculated with common wheat seeds to evaluate their plant growth promoting activity. Cultivable population of Bacillus and Pseudomonas were 2.20 × 10^6 and 6.98 × 10^4 CFU g^{-1} dry matter in mesophilic digestate, while were 6.86 × 10^5 and 5.65 × 10^4 CFU g^{-1} dry matter in thermophilic digestate. Twenty-five bacterial isolates from mesophilic digestate and 12 bacterial isolates from thermophilic digestate showed positive plant growth promoting traits or antifungal activity. In plant growth promoting assay, all isolates significantly promoted growth of wheat seedlings ($p < 0.05$). Seedlings stem length was increased from 28.5% to 38.6% by bacteria inoculation. In addition, bacteria inoculation increased seedlings stem weight from 113.3% to 214.2% and root weight from 108.6% to 207.2% as compared to un-inoculated control. The results showed that anaerobic digestate was a potential source for isolation of PGPB, and PGPB in digestate would be beneficial for plant growth with fertilizer application.*

Keywords— Anaerobic digestate, Plant growth promoting bacteria (PGPB), Bacillus, Pseudomonas, Common wheat (Triticum aestivum).

I. INTRODUCTION

Anaerobic digestion of organic wastes produces biogas and a nutrient-rich digestate. Digestate contains partially-degraded organic matter, inorganic plant nutrients and microbial biomass, therefore it can be used as soil conditioner or fertilizer on agricultural field (Alburquerque et al., 2012). The use of digestate as a fertilizer is considered eco-friendly since it recycles plant nutrients in the organic waste and thus reduces large scale use of chemical fertilizers. Furthermore, plant nutrients are present in inorganic plant-available forms in digestate at a markedly higher level compared to undigested organic wastes, because of the mineralization of organic nutrients during anaerobic digestion process (Umetsu et al., 2002). Previous researches have documented the beneficial effects of digestate as organic fertilizer on plant growth and nutrients uptake, and soil structure and microbial activity (Muscolo et al., 2017; Risberg et al., 2017; Solé-Bundó et al., 2017; Tampio et al., 2016).

Plant growth promoting bacteria (PGPB) represent a wide variety of bacteria, which occupy the rhizosphere of many plant species and promote host plant growth directly by solubilizing minerals such as phosphorus, producing siderophores that chelate iron and producing phytohormones (Grobelak et al., 2015). Phosphorus (P) is one of the major macronutrients required for growth and development of plant. Generally, soils have large reserves of total P, but the amount available to plants is low as majority of soil P is found in insoluble forms (Ahemad and Kibret, 2014; Vessey, 2003). PGPB could make phosphorus available to plants by solubilizing and mineralizing inorganic and organic phosphorus in soils (Ahemad and Kibret, 2014). Iron is also an essential nutrient plant growth. However, iron exists mainly as Fe^{3+} in aerobic environment and is likely to form insoluble hydroxides and oxyhydroxides which are not unavailable to plants (Rajkumar et al., 2010). The siderophores, which are low-molecular mass iron chelators, secreted by some PGPB could solubilize iron from minerals or organic compounds under conditions of iron limitation to make iron accessible to plants (Indiragandhi et al., 2008). Indole-3-acetic acid (IAA) is the primary phytohormone produced by RGPB and has various effects on plant growth promotion such as cell division and elongation, stimulation of seed germination, and increase root development (Ahemad and Kibret, 2014). PGPB can also stimulate plant growth indirectly by suppressing phytopathogens in forms of producing antibiotics, siderophores, and fungal cell wall-lysing enzymes (Ji et al., 2014). The largest groups of PGPB are *Pseudomonas*, *Bacillus*, *Enterobacter*, and *Erwinia* (Grobelak et al., 2015). Majority of researched PGPB are isolated from rhizosphere and they are

generally known as plant growth promoting rhizobacteria (PGPR) (Khalid et al., 2004). However, anaerobic digestates are host to numerous PGPB and little attention has been focused on the isolation and characterization of PGPB from anaerobic digestate.

In the present study, two groups of PGPB: *Bacillus* and *Pseudomonas* isolated from two types of anaerobic digestate were screened on plant growth promoting traits including phosphate solubilization, siderophore production and phytohormone production, as well as antifungal activity. Selected bacterial isolates were further evaluated for their growth promoting activity on common wheat (*Triticum aestivum*).

II. MATERIAL AND METHOD

2.1 Anaerobic digestate samples collection

Anaerobic digestate samples were collected from two continuously stirred tank reactors (CSTR) (Yamashiro et al., 2013) operated at mesophilic (37°C) and thermophilic (55°C) temperatures. Mesophilic and thermophilic digesters were fed daily with dairy manure. To ensure homogeneity of samples, digesters were thoroughly stirred before digestate samples were collected. Mesophilic and thermophilic digestates collected from the digesters were thereafter referred to as MAD and TAD, respectively. Digestate samples were immediately kept at 4°C and isolation of bacteria was done within 24 h.

2.2 Isolation of bacteria from anaerobic digestate samples

Bacillus and *Pseudomonas* were isolated by the spread plate method. Samples were diluted 10-fold with phosphate buffered saline (pH 7.4), and 100 µl of diluent was spread on BD BBL™ MYP (BD Falcon™, Franklin Lakes, NJ, USA) plates to isolate *Bacillus*, and Difco™ Cetrimide Agar Base (Becton, Dickinson and Company, Sparks, MD, USA) plates to isolate *Pseudomonas*, respectively. After incubation, typical colonies were counted and calculated as colony forming units per gram of dry matter (CFU g^{-1} dry matter). Then one-hundred *Bacillus* isolates and one-hundred *Pseudomonas* isolates of each digesate sample were selected randomly and maintained on the LB agar plates for further analyses.

2.3 Screening of bacterial isolates for plant growth promoting traits and antifungal activity

Phosphate solubilization ability of bacterial isolates was determined with a Pikovskaya's agar plate (HiMedia Laboratories Ltd, Mumbai, India). Bacterial strains were spotted on Pikovskaya's agar plate and incubated at 28°C for 3 days. The isolates which produced a halo zone around the colony was determined as having ability to solubilize phosphate.

Chrome Azurol Sulphonate (CAS) assay was used to detect siderophore production of bacterial isolates. The CAS agar plate was made according to method described by Lakshmanan et al. (2015). Bacterial isolates were spotted on CAS agar and incubated at 28°C for 3 days. Formation of orange halo around the colonies confirmed the production of siderophore.

IAA (indole-3-acetic acid) production of bacterial isolates was determined according to the method previously described by Ji et al. (2014). Bacterial strains were inoculated into 5 ml LB broth with 0.1% (w/v) L-tryptophan and incubated on a rotary shaker at 150 rpm for 3 days at 30°C. The cultures were centrifuged at 10,000 rpm for 10 min at 4°C to obtain a supernatant. The supernatant (2 ml) was mixed with 4ml of Salkowski's reagent (2 ml 0.5 M $FeCl_3$ and 98 ml 35% perchloric acids) and incubated for 25-30 min in the dark at room temperature. The development of a pink color indicates IAA production, and optical density of mixtures was read at 530 nm with a spectrophotometer (NanoDrop2000c, Thermo Scientific). The concentrations of IAA produced per milliliter of culture (µg ml^{-1}) were estimated by comparison with a standard curve of IAA in the range of 0.5-100 µg ml^{-1}.

Antifungal activity of bacterial isolates was tested using the dual culture method with Potato Dextrose Agar (PDA, Becton, Dickinson and Company, Sparks, MD, USA). In this study, the fungal strain *Fusarium nivale* f. sp. g*raminicola* (MAFF 235153) purchased from National Institute of Agrobiological Sciences, Japan (NIAS; Tsukuba, Japan) was used. The fungal mycelia were inoculated in the center of a PDA agar plate and incubated for 24 h at 25°C followed by inoculation of the isolates 3 cm away from the center of the PDA plate. The fungal mycelium alone was inoculated as a control. After incubation at 28°C for 7 days, the antifungal activity was measured by the percent of inhibition of growth (PGI): PGI = (1 – R/Rc) × 100%, where R represents the radius of the fungal mycelia in the plate inoculated with bacteria isolates, and Rc represents the radius of the fungal mycelia in the control plate.

2.4 Identification of bacteria isolates

For identification of bacterial isolates, Bruker microflex mass spectrometer system (microflex LT/SH, Bruker Daltonics, Kanagawa, Japan) was used. Two methods, direct smear method and on-plate extraction method were used in this study. For

the former method, bacterial colony was directly smeared onto a spot on polished steel MALDI target plates using sterile toothpicks. Thin spots of bacteria were then dried in a safety cabinet, and subsequently overlaid with 1µl of the matrix solution, comprising a HCCA (α-Cyano-4-hydroxycinnamic acid) matrix (Bruker Daltonik) for 5 min. For the on-plate extraction method, an extraction step by 1µl of 70% formic acid (Wako Pure Chemical Industries, Osaka, Japan) was introduced before cocrystallization with the matrix. *Escherichia coli* (K-12, laboratory stock) was used as a positive and quality control, and formic acid and the matrix was used as negative control at each run. The Bacterial Test Standards (Bruker Daltonics) was used for instruments calibration with each run. The samples prepared by each method were subjected to the microflex mass spectrometer, and results were analyzed by MALDI Biotyper 3.0 software (Bruker Daltonics).

2.5 Plant growth promoting assay with common wheat (*Triticum aestivum*)

Plant growth promoting assay with common wheat was conducted as described by Grobelak et al. (2015). The seeds of common wheat (*Triticum aestivum*) were surface sterilized with 1.5% (v/v) sodium hypochlorite for 10 min and washed with sterile water for 3 times. Subsequently, sterilized seeds were planted in plastic pots filled with 100g of commercial soil which was sterilized by autoclave. Bacterial isolates were incubated in LB broth at 30°C for 3 days and 150 rpm in a rotary shaker. Then, the bacterial cultures were centrifuged at 6000 rpm for 10 min, cell pellets were suspended in sterile water and densities were adjusted to 1×10^8 CFU ml^{-1}. The bacterial suspensions were applied immediately after seeding with 1 ml pot^{-1}. Only sterile water was applied as control. Pots were maintained at room temperature (26-28°C) for 4 weeks with five replicates, and then stems and roots of the plants were weighed for biomass determination and length of the plants was also measured.

2.6 Statistical analysis

Results are expressed as mean values ± standard deviation. Data from plant growth promoting assay were statistically analyzed by analysis of variance (ANOVA) with treatment means separated by Tukey test at $p < 0.05$ using SAS Statistical Software version 9.4 (SAS Institute Inc., USA).

III. RESULTS AND DISCUSSION

3.1 Isolation and characterization of bacteria for plant growth promoting traits and antifungal activity

Cultivable population of *Bacillus* and *Pseudomonas* were 2.20×10^6 and 6.98×10^4 CFU g^{-1} dry matter in MAD, which were higher than 6.86×10^5 and 5.65×10^4 CFU g^{-1} dry matter in TAD. Then 100 *Bacillus* isolates and 100 *Pseudomonas* isolates were selected from each digestate sample and screened for plant growth promoting traits and antifungal activity. The results are presented in Table 1. Twelve *Bacillus* isolates (12%) from the MAD showed siderophores production and antifungal activity, in which 5 isolates also showed IAA production. Thirteen *Pseudomonas* isolates (13%) showed siderophores and IAA production, in which only one isolate showed phosphate solubilization. For *Bacillus* isolates from TAD, only 5 isolates (5%) were positive for plant growth promoting traits or antifungal activity, and 7 *Pseudomonas* isolates (7%) produced IAA in which 6 isolates also showed siderophores production.

It is known that anaerobic digestion process inactivates bacteria in feedstock due to many factors, such as reactor temperature, feedstock retention time, and digestate pH (Smith et al., 2005; Wagner et al., 2008). Thermophilic temperature causes greater inactivation of bacteria than mesophilic temperature (Iwasaki et al., 2011), which explains higher cultivable bacteria and percent of PGPB observed in MAD than in TAD.

TABLE 1
NUMBER OF BACTERIAL ISOLATES SHOWED PLANT GROWTH PROMOTING TRAITS AND ANTIFUNGAL ACTIVITY FROM ANAEROBIC DIGESTATES

Sample	Bacterial genus	Phosphate solubilization	Siderophores production	IAA production	Antifungal activity
MAD	*Bacillus*	0	12	5	12
	Pseudomonas	1	13	13	0
TAD	*Bacillus*	0	4	3	5
	Pseudomonas	0	6	7	0

MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate; IAA: Indole-3 acetic acid.

For plant growth promoting assay, 6 bacterial isolates were selected and their plant growth promoting traits and antifungal activity are presented in Table 2. Bacteria capable of phosphate solubilization are known to promote plant growth by increasing phosphorous uptake. The phosphate solubilizing isolate (MAD-21) was identified as *Pseudomonas putida*. Similarly, phosphate solubilizing ability of *Pseudomonas putida* has been reported in previous studies (Malboobi et al., 2009; Pandey et al., 2006). Fluorescent pseudomonads are considered to be one of the most promising groups of PGPB (Bhattacharyya and Jha, 2012). In this study, fluorescent pseudomonads isolate (MAD-17) showed siderophores production and IAA production of 17.3 μg ml^{-1}, similar plant growth promoting traits of fluorescent pseudomonads were reported by Saber et al. (2015).

TABLE 2
BACTERIAL ISOLATES SELECTED FOR PLANT GROWTH PROMOTING ASSAY

Bacterial isolate no.	Phosphates solubilization	Siderophores production	IAA production (μg ml^{-1})	Antifungal activity (PGI%)	Identification
MAD-05	-	+	1.06 ± 0.03	62.75 ± 2.45	*Bacillus subtilis*
MAD-17	-	+	17.3 ± 1.47	-	fluorescent pseudomonads
MAD-21	+	+	18.43 ± 1.0	-	*Pseudomonas putida*
TAD-05	-	-	11.7 ± 1.18	43.53 ± 2.35	*Bacillus licheniformis*
TAD-11	-	+	11.59 ± 0.28	-	*Pseudomonas* spp.
TAD-12	-	-	24.54 ± 1.24	-	*Pseudomonas aeruginosa*

MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate.
Phosphate solubilization (+); non phosphate solubilization (-). Siderophores production (+); non siderophores production (-). IAA: Indole-3 acetic acid; values are expressed as means ± standard deviation. PGI: percent of growth inhibition; values are expressed as means ± standard deviation; non growth inhibition (-).

The production of phytohorrnones by bacteria is one of the most important factors of plant growth promotion (Ahemad and Kibret, 2014). Khalid et al. (2004) have categorized IAA-producing bacteria into three principal groups: lower producers (1 to 10 μg ml^{-1}), medium producers (11 to 20 μg ml^{-1}) and higher producers (21 to 30 μg ml^{-1}). Among 6 isolates for plant growth promoting assay, MAD-05 (*Bacillus subtilis*) was lower IAA producer (1.06 μg ml^{-1}), and TAD-12 (*Pseudomonas aeruginosa*) produced highest amount of IAA (24.54 μg ml^{-1}), which was higher producer. The rest of isolates were medium producers (Table 2).

Biological control, or biocontrol means to control plant diseases by application of microorganisms, which is an environmental-friendly and efficient disease management approach (Ahemad and Kibret, 2014). In this study, *Bacillus* isolates (MAD-05, *Bacillus subtilis* and TAD-05, *Bacillus licheniformis*) showed high antifungal activity of 62.75% and 43.53% PGI, which may contribute to the competition for space and nutrients and secretion of antifungal compounds (Yang et al., 2015). Similarly, *Bacillus* species have been widely reported to have antifungal activity against a wide variety of phytopathogens (Ji et al., 2014; Kumar et al., 2012; Liu et al., 2016). Therefore, further application of *Bacillus* isolates as biocontrol agents could be expected.

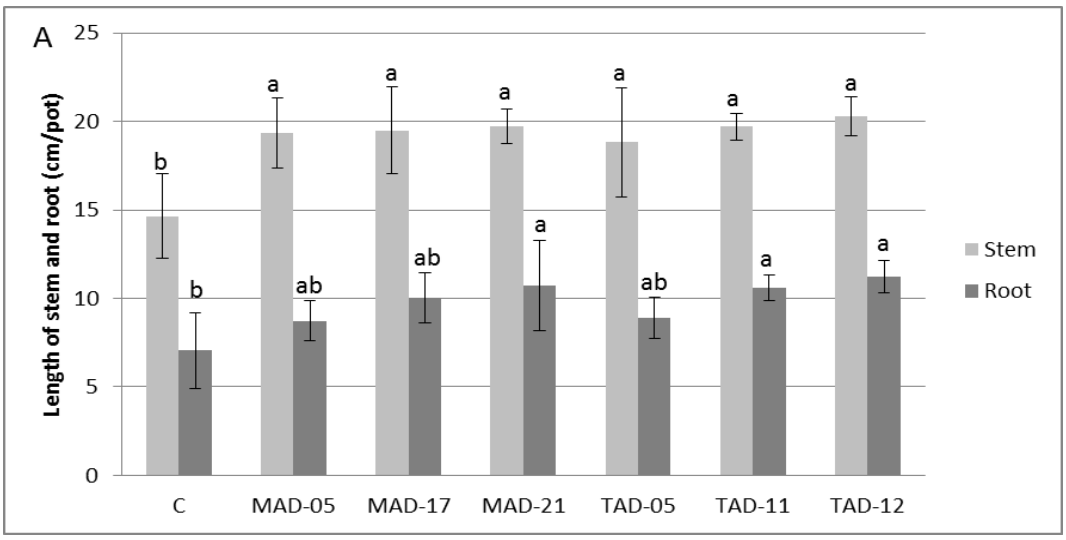
3.2 Effect of bacteria inoculation on plant growth of common wheat (*Triticum aestivum*)

The effects of selected bacterial isolates inoculation on plant growth were evaluated with common wheat (Fig. 1 and 2). Stem length of the seedlings inoculated with bacterial isolates (Fig. 2A) significantly increased from 28.5% to 38.6% compared to those of un-inoculated control ($p < 0.05$), and the differences between each treatments were non-significant ($p > 0.05$). Inoculation with MAD-21 (*Pseudomonas putida*), TAD-11 (*Pseudomonas* spp.) and TAD-12 (*Pseudomonas aeruginosa*) also significantly ($p < 0.05$) increased 51.8%, 50.1% and 59.21% of root length (Fig. 2A). The bacterial isolates inoculation further increased biomass of seedlings stem and root (Fig. 2B). Inoculation with TAD-12 (*Pseudomonas aeruginosa*) showed the highest increases in stem and root weight (214.2% and 207.2%, respectively) of the seedlings. After the TAD-12, other 5 bacterial isolates inoculation increased stem weight from 113.3% to 163.6%, and root weight from 108.6% to 160.1% compared to un-inoculated control ($p < 0.05$).

FIG.1. PLANT GROWTH PROMOTING ASSAY WITH COMMON WHEAT. C: UNTREATED CONTROL; MAD-05: *BACILLUS SUBTILIS*; MAD-17: FLUORESCENT PSEUDOMONADS; MAD-21: *PSEUDOMONAS PUTIDA*; TAD-05: *BACILLUS LICHENIFORMIS*; TAD-11: *PSEUDOMONAS* SPP.; TAD-21: *PSEUDOMONAS AERUGINOSA*.

The inoculation of plants with PGPB increased plants length of stem and root, these results were agreement with observation of Balseiro-Romero et al. (2017) and Grobelak et al. (2015). It is well-known that inoculation with IAA-producing bacteria increases plant growth by promoting root growth and length, resulting in greater root surface area which enables the plant to absorb more nutrients from soils (Vessey, 2003). Inoculation with TAD-12 (*Pseudomonas aeruginosa*) showed the highest promotion in stem and root weight, which can be related with the highest production of IAA observed in the isolates (Table 3). Similarly, several researches have demonstrated that *Bacillus* and *Pseudomonas* strains produced IAA and are able to regulate root development (Ji et al., 2014; Kumar et al., 2012; Scagliola et al., 2016; Son et al., 2014).

It has been suggested that the performance of PGPB could be enhanced through the use of PGPB mixtures, and Dary et al. (2010) and Malboobi et al. (2009) have demonstrated that inoculation with mixed PGPB can promote plant growth more than a single strain. Although the effects of mixed PGPB inoculant were not investigated in this study, it could be expected that digestate is an inoculant of PGPB mixtures and promote plant growth more effective than single bacterial strain inoculant.



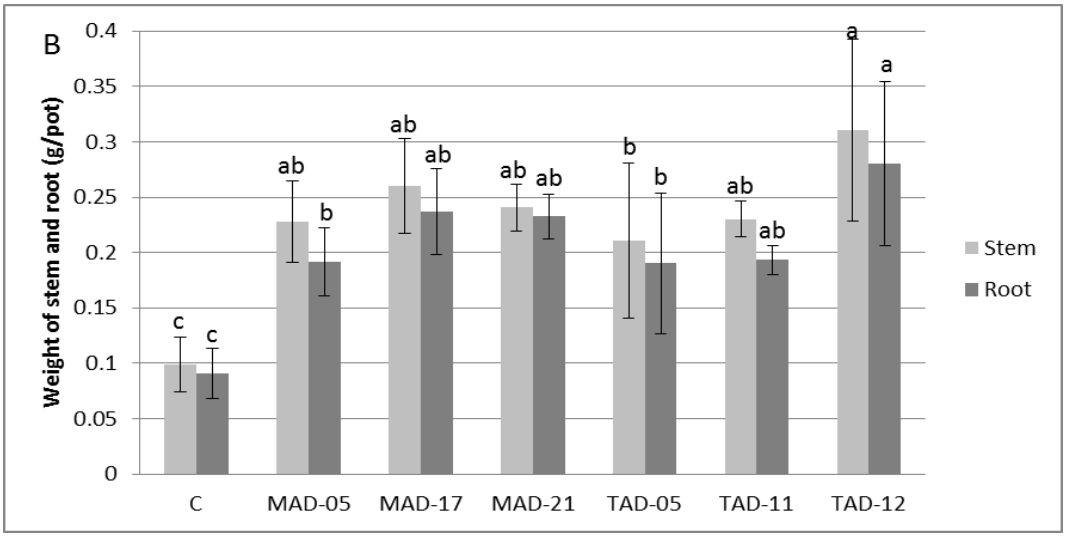


FIG.2. STEM AND ROOT LENGTH (A) AND WEIGHT (B) OF COMMON WHEAT SEEDLINGS INOCULATED WITH BACTERIAL ISOLATES IN PLANT GROWTH PROMOTING ASSAY. C: UNTREATED CONTROL; MAD-05: *BACILLUS SUBTILIS*; MAD-17: FLUORESCENT PSEUDOMONADS; MAD-21: *PSEUDOMONAS PUTIDA*; TAD-05: *BACILLUS LICHENIFORMIS*; TAD-11: *PSEUDOMONAS* SPP.; TAD-21: *PSEUDOMONAS AERUGINOSA*.

IV. CONCLUSION

In conclusion, anaerobic digestate is a large reservoir of bacteria capable of promoting plant growth. In this study, plant growth promoting *Bacillus* and *Pseudomonas* were isolated and characterized from mesophilic and thermophilic digestates. Two types of digesates contained different cultivable bacteria and percent of PGPB which may be attributed to the different operation temperature of digesters. Bacterial isolates showed plant growth promoting traits including phosphate solubilization, siderophores production and IAA production. The selected bacterial isolates significantly promoted plant growth, which is most probably due to their ability to produce IAA. These isolates can be applied as inoculants for improving plant growth. *Bacillus* isolates from digestates showed antifungal activity, therefore, it will be important to perform further studies investigating their antifungal activity in field experiments.

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