Assessment of Fertilizer Properties of Digestate from Anaerobic Digestion of Dairy Manure

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Qi Guangdou

Doctoral Program in Animal and Food Hygiene

Graduate School of Animal Husbandry

Obihiro University of

Agriculture and Veterinary Medicine

乳牛ふん尿の嫌気消化液の肥料特性評価

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Abstract

The large amount of livestock manure and slurry produced from livestock husbandry has a potential hazard source to the environment and public health, if they are improperly managed or treated. Treatment of these organic wastes in biogas plants (BGPs) with a biochemical technology; anaerobic digestion (AD), is considered the most suitable disposal because it recycles organic wastes, produces renewable energy, reduces greenhouse gas (GHG), and provides valuable bio-fertilizers. Recently, BGPs receive much attention. As a result, a lot of BGPs have been installed worldwide. With the development of BGPs, the amount of anaerobic digestate (digested residue after AD process) also increased sharply. The sustainability of full scale BGPs depends highly on the appropriate disposal of anaerobic digestate.

In Hokkaido, Japan, 330 BGPs are now in operation and anaerobic digestate from these BGPs is mostly used as a fertilizer for agricultural field. Anaerobic digestate contains large of plant nutrients, especially in inorganic plant-available forms, which could be used to reduce the consumption of mineral fertilizers. Generally, AD process can be conducted under mesophilic or thermophilic temperatures. Mesophilic digestion requires lower energy cost with a higher stability process, while thermophilic digestion leads to more rapid digestion and a higher reduction rate of pathogen. However, little is known about their effects on the fertilizer properties of digestate.

Agricultural application of anaerobic digestate has caused public concern in recent years due to the risk related to transportation of pathogenic bacteria and heavy metals to the environment. In addition, the effect of microorganisms in organic fertilizers has recently attracted attention, especially for the plant growth promoting effects of *Bacillus* and *Pseudomonas* species, which have been widely researched. Plant growth promoting bacteria (PGPB) can occupy the rhizosphere of many plant species and have beneficial effects on plant growth directly by assisting in nutrients acquisition or providing phytohormones, or indirectly decreasing inhibitory effects of various fungal pathogens. However, anaerobic digestate is a host to numerous PGPB and little attention has been focused on the isolation and characterization of PGPB from anaerobic digestate.

Therefore, this PhD thesis was focused on two main objectives: to investigate fertilizer properties of mesophilic and thermophilic digestates from livestock manure for plant nutrient contents, a special attention was given to plant growth promoting bacteria (PGPB); and to evaluate the environmental risks related to pathogenic bacteria and heavy metal contents.

In Chapter 1, mesophilic and thermophilic digestates from laboratory scale anaerobic digesters were collected for the analysis of plant nutrients, which were N and NH_4^+-N, P (P_2O_5), K (K_2O), Ca (CaO), and Mg (MgO). For environmental risks, pathogenic bacteria (*Salmonella*, *Campylobacter*, *Escherichia coli,* and *Enterococcus*) and heavy metals (Mn, Zn, Cu, and Ni) were investigated. The results show that the two digestates contained similar amount of plant nutrients, while thermophilic digestate had higher NH_4^+-N content (12.2 g/kg) than that of mesophilic digestate (9.8 g/kg). The contents of pathogenic bacteria and heavy metals were analyzed to determine their environmental risk. The reduction rates of pathogenic bacteria were above 90% in the thermophilic digestate, and the maximum rate was 99.7% for *E. coli*, which was higher than that in mesophilic digestate (a minimum of 73.2% for *Campylobacter* and maximum of 96.9% for *E. coli*), which indicates that thermophilic digestate has a lower risk to the environment. Lower levels of heavy metals were detected in digestates from dairy manure than those in other feedstocks.

In Chapter 2, plant growth promoting *Bacillus* and *Pseudomonas* were isolated from mesophilic and thermophilic digestates and characterized. Three different plant growth promoting activities, which are phosphate solubilization ability, siderophore production and phytohormone production, as well as antifungal activity were selected and 200 bacteria were isolated from each digestate. The isolated bacteria, based on plant growth promoting traits, were selected and inoculated with common wheat seeds to evaluate their plant growth promoting activities. The results showed that *Bacillus* in dairy manure increased significantly after anaerobic digestion. 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. 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.

Chapter 3 was focused on the bacterial load (plant growth promoting bacteria and pathogenic bacteria) in anaerobic digestates from two full scale biogas plants (BGPs) in Hokkaido. Anaerobic digestate samples were collected from feedstock tank, fermentation tank, sterilization tank and storage tank at Mikage biogas plant. In Shikaoi biogas plant, anaerobic digestate samples were only collected from feedstock tank and storage tank. The results showed that *Bacillus* in feedstock decreased after anaerobic digestion in full scale BGPs, which was different from the results of chapter 2. Furthermore, pathogenic bacteria, except *Campylobacter*, were eliminated. These results indicated that there was a difference in bacteria reduction rate between laboratory scale and full scale anaerobic digestion. However, *Bacillus* was detected at a high level in two digestates from BGPs, which indicates that digestates may be a potential bio-fertilizer. On the other hand, *Campylobacter* residue was detected after both laboratory scale and full scale anaerobic digestion, which was considered a possible source of environmental contamination.

The results from this PhD thesis show that (1) operating temperature of AD process is the major determinant factor that affects the fertilizer properties of anaerobic digestate. High temperature leads to high contents of inorganic plant-available nutrients (NH_4^+-N) and high reduction rate of pathogenic bacteria after AD process. However, higher cultivable bacteria and higher percent of PGPB were observed in mesophilic digestate than that in thermophilic digestate. (2) Anaerobic digestate is a large reservoir of bacteria capable of promoting plant growth. These bacteria were able to colonize the rhizosphere with digestate application and could increase the availability of nutrients for plant and decrease disease symptoms, which make digestate an effective biofertilizer. (3) The heavy metals in anaerobic digestate are likely to show an increased risk to the environment. In this study, the heavy metal concentrations of digestates were lower than in other feedstock, but not decreased. Therefore, it is imperative to remove these heavy metals before the application of anaerobic digestate, especially when the feedstock used, such as sewage sludge, contains

high contents of heavy metal. (4) In full scale biogas pants, all detected bacteria were reduced to undetectable level expect *Bacillus* and *Campylobacter*. The presence of *Bacillus* also makes anaerobic digestate a potential bio-fertilizer. However, *Campylobacter* residue is considered a possible source of environmental contamination.

General Introduction

1. Anaerobic digestion

In recent decades, there has been concern regarding environmental problems and public health associated with livestock manure treatment. Along with the intensive development of animal husbandry, livestock manure production has increased dramatically. When untreated or not managed properly, livestock manure becomes a potential source of hazard to the environment and public health (Holm-Nielsen et al., 2009; Yamashiro et al., 2013).

Anaerobic digestion (AD) is a promising process to treat organic wastes, including livestock manure, resulting in two products: biogas and a digested residue called digestate. Organic compound degrading bacteria in feedstock convert carbohydrates, peptides, polysaccharides and lipids into methane (CH_4) and carbon dioxide (CO_2). The flow of the process of the degradation of organic matter during anaerobic digestion is illustrated in Fig. 1. In the hydrolysis stage, polymers such as carbohydrates, proteins and fats are hydrolyzed and converted into monomers (simple sugars, amino acids and long-chain fatty acids). Then monomers are converted into short-chain fatty acids, mainly formic acids, propionic acids and butyric acids in the acidogenesis stage. In the last stage, short-chain fatty acids are converted into acetic acids, which are used to produce biogas during methanogenesis stage. Biogas contains 50-70% of methane and 30-50% of carbon dioxide, and can be used for heat and electricity generation, or further compressed to bio-methane for vehicle fuel (Risberg et al., 2017; Zhou et al., 2016).

AD of livestock manure could be conducted under mesophilic (30 to 38°C) or thermophilic (49 to 57°C) conditions. In general, mesophilic anaerobic digestion is more widely used as its lower energy cost and higher stability of the process. However, interest in thermophilic anaerobic digestion has been raised as low gas yield and residual of pathogen for mesophilic digestion. Thermophilic digestion is shown to allow higher organic loading rates (OLR) and be more effective on reducing pathogenic and antibiotic resistant bacteria (Gavala et al., 2003; Min et al., 2016). Table 1 shows the difference of anaerobic digestion process of organic wastes at different temperature ranges.

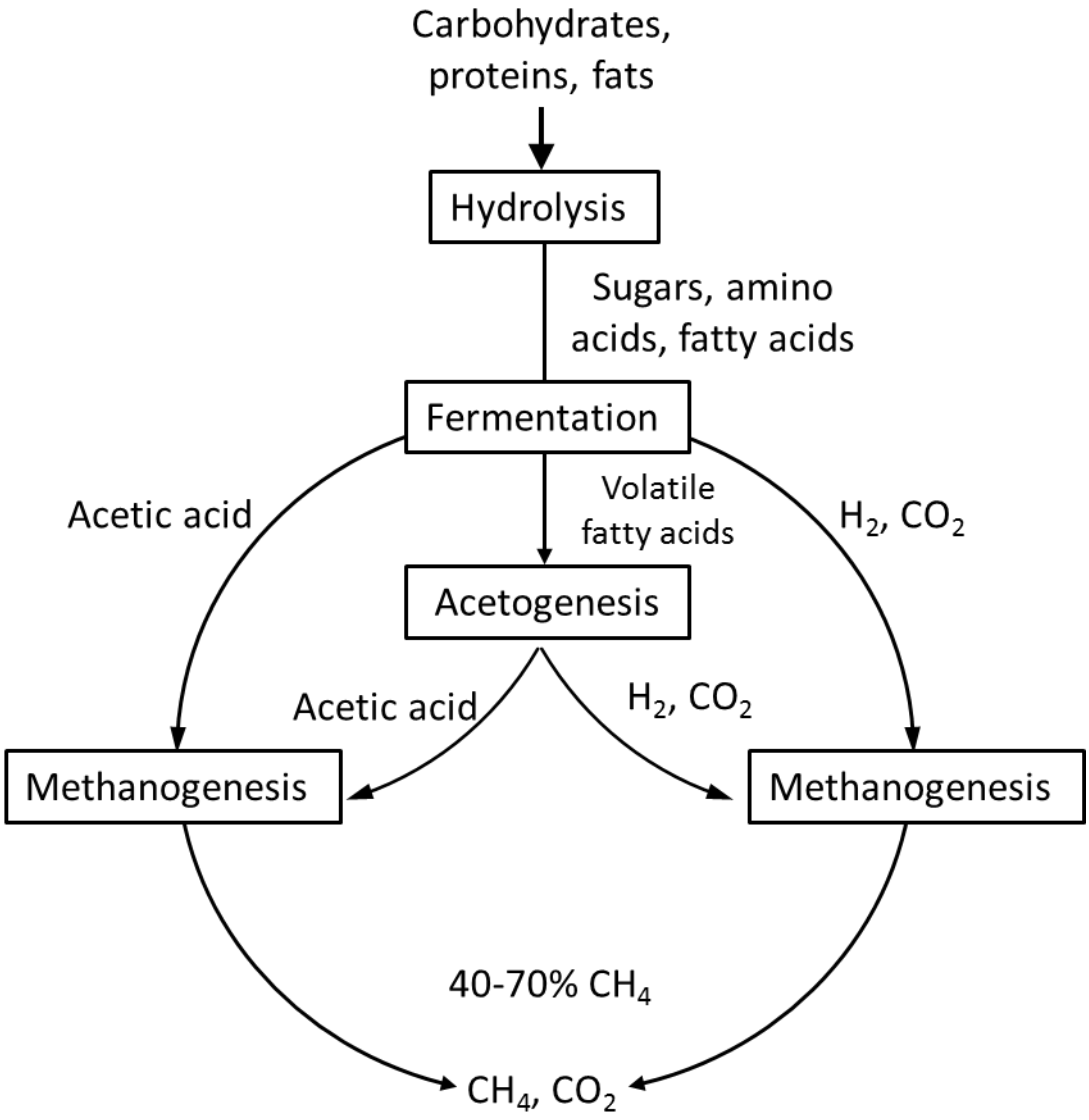

Figure 1: Process flow of the degradation of organic matter through anaerobic digestion. Source: (Li et al., 2011)

Table 1: Performance comparison of mesophilic and thermophilic anaerobic digestion of organic wastes

	Mesophilic digestion	Thermophilic digestion
Temperature	30 - 38°C	49 - 57°C
Degradation rate	Slow	Fast
Gas generation rate	Slow	Fast
Organic loading rate	2.0 ~ 3.0 kg/m3/day	5.5 ~ 6.5 kg/m3/day
Hydraulic retention time	20 ~ 30 day	10 ~ 20 day
Sanitization risk	High	Low
Energy consumption	Low	High

2. Anaerobic digestate utilization

Anaerobic digestate is a mixture of degraded organic compounds, inorganic macronutrients and microbial biomass (Alburquerque et al., 2012). Furthermore, macronutrients 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 that are found in feedstock during anaerobic digestion (Umetsu et al., 2002). For example, the ammonium (NH_4^+-N) concentration, which is more readily available for crops than organic nitrogen, is significantly higher in digestate than in feedstock (Massé et al., 2007; Riva et al., 2016; Umetsu et al., 2002). Therefore, digestate is commonly used as an organic fertilizer and an amendment to agricultural soil. Utilization of anaerobic digestate serves to recycle macronutrients and reduce the consumption of fossil fuel-dependent mineral fertilizers (Holm-Nielsen et al., 2009). The flow chart of anaerobic digestate cycle as bio-fertilizer is illustrated in Fig. 2.

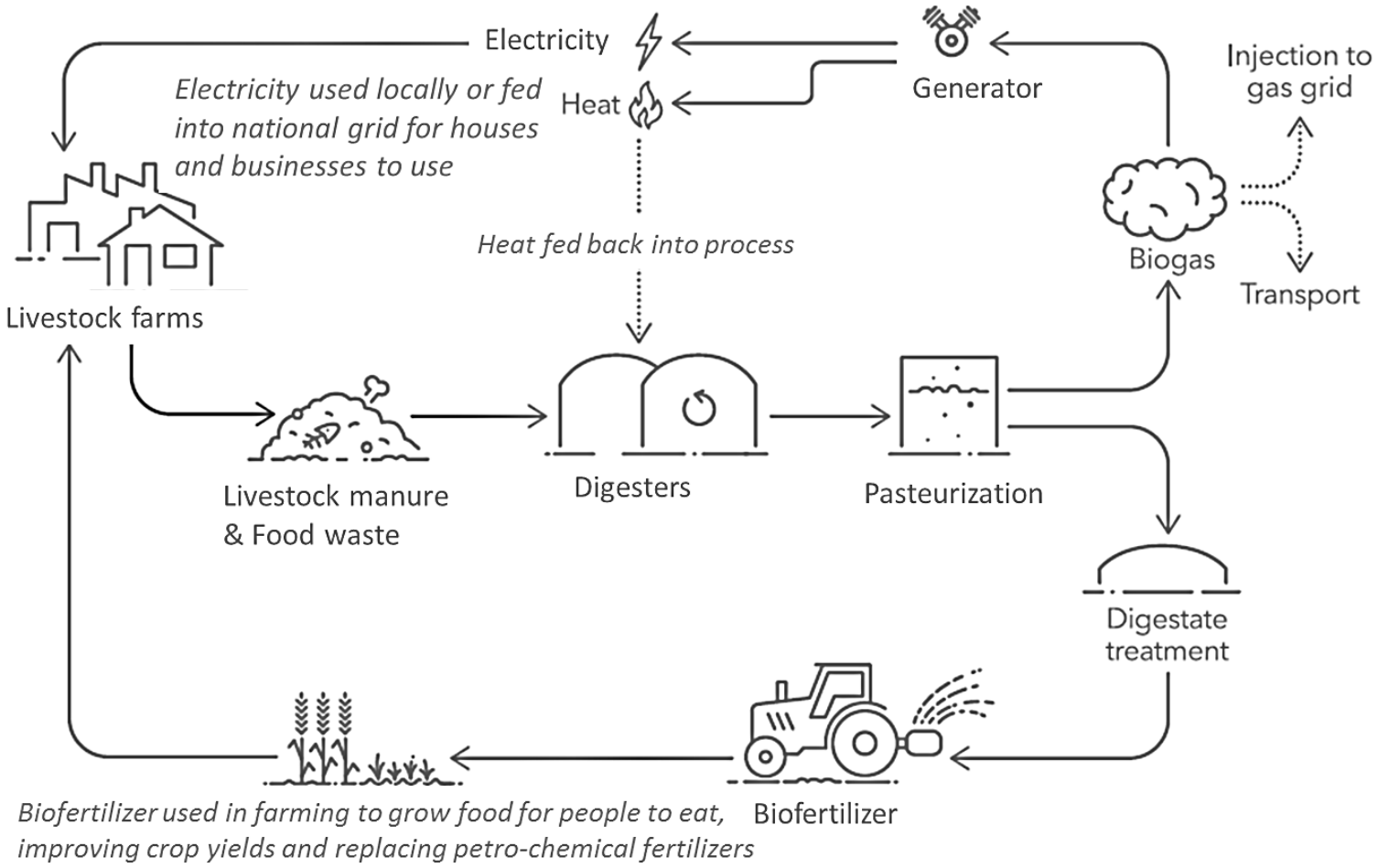

Figure 2: Flow chart of anaerobic digestate cycle as bio-fertilizer. Source: (TAMAR ENERGY LIMITED, LONDON)

The anaerobic digestate composition and fertilizer property can be highly various depending on the feedstock types and AD process operating conditions (Alfa et al., 2014; Solé-Bundó et al., 2017). In recent years, fertilizer properties of digestate from different feedstocks have been widely investigated (Abubaker et al., 2012; Alburquerque et al., 2012; Risberg et al., 2017), but little is known about the effect of operating conditions. As mentioned earlier, AD process could be conducted under different temperatures (mesophilic and thermophilic). Mesophilic digestion requires lower energy cost and shows a higher stability of the process, while thermophilic digestion leads to more rapid digestion rate and higher reduction rate of pathogen. However, much is not known about their effects on fertilizer properties of digestate.

3. Environmental risks with digestate application

On the other hand, agricultural application of anaerobic digestate is not environment ally risk-free since it may introduce many chemical and biological contaminants into soils (Nkoa, 2014). In recent years, agricultural application of anaerobic digestate has caused public concern due to its increased heavy metal content (Dong et al., 2013). The reason for the increasing concern is that heavy metals are generally used as feed additives to promote livestock growth, and their contents are found to be increasing in livestock manure, which is used as a feedstock for anaerobic digestion (Zhu and Guo, 2014). Therefore, the contents of heavy metals in the digestate should be considered when applied to the soils. In addition, the available information on the biological property of anaerobic digestate other than fermentative (degradative) bacteria is limited. It is well known that livestock manure contains many pathogenic and non-pathogenic bacteria, such as *Pseudomonas*, *Klebsiella*, *Salmonella*, *Bacillus*, *Shigella*, *Clostridium*, and other microorganisms, which may survive during the AD process and persist in digestate (Alfa et al., 2014; Owamah et al., 2014). Biological contaminants in digestate, such as pathogenic bacteria, are of great concern to the public as it they increase the risk of pathogen spread. Pathogenic bacteria are reduced during the AD process, but the reduction rate depends on many factors, such as the fermentation temperature, fermentation time, and initial number of bacterial species in the

feedstock (Sahlström, 2003). Therefore, many pathogenic bacteria may still be present in digestate and cause a health risk for both people and animals.

4. Microorganisms related to anaerobic digestion process

AD process is the biological treatment of organic matter in the condition of no oxygen, offering the benefits of reducing treatment cost and environmental pollution and producing eco-friendly energy as biogas. The AD process has attracted considerable attention in the past two decades, and knowledge of microbiological aspects of the process has also accumulated significantly (Narihiro and Sekiguchi, 2007). Fig. 3 shows the anaerobic digestion process of the degradation of organic matter and microorganism involved in the process.

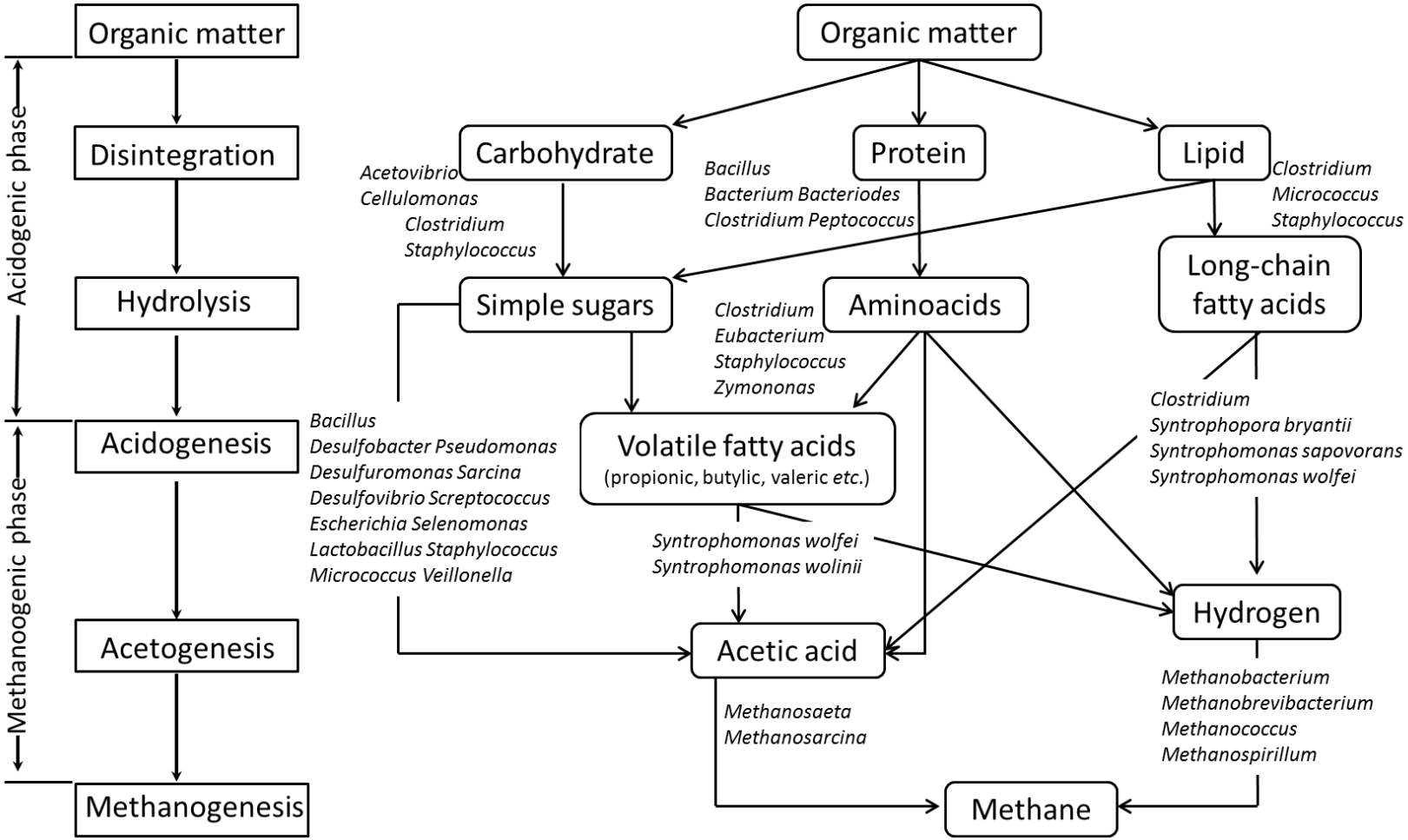

Figure 3: Anaerobic digestion process of the degradation of organic matter and microorganisms involved in the process. Source: (Noike et al., 2009)

5. Plant growth promoting bacteria (PGPB)

Plant growth promoting bacteria (PGPB) are the soil bacteria inhabiting around the rhizosphere and promoting plant growth in direct or indirect mechanisms which is shown in Fig. 4. Generally, PGPB promote the plant growth directly by either supplying the plant with nutrients (nitrogen, phosphorus and essential mineral) or producing phytohormones, or indirectly by decreasing the inhibitory effects of fungal phytopathogens on plant growth in the forms of biological control (Ahemad and Kibret, 2014; Vessey, 2003).

Nitrogen (N) is the necessary nutrient for all plants which is used to synthesize bio-molecules such as proteins and nucleic acids. However, approximately 78% atmospheric nitrogen (N_2) is unavailable to the most growing plants. Biological nitrogen fixation (BNF) is the process which changes nitrogen to ammonia (NH_3) by nitrogen fixing microorganisms through nitrogenase, a highly conserved enzyme (Ahemad and Kibret, 2014; Goswami et al., 2016). Phosphorus is a macronutrient that is required by plants, but the available rate of phosphorus in soil is very low due to the immobilization of phosphate by mineral ions, such as Fe (II) and Ca (II). Some PGPB can facilitate the conversion of insoluble phosphorus in soil to plant-available forms (Rodríguez and Fraga, 1999). Iron, which also mainly exists in insoluble forms in soil, is another essential nutrient for plants. Siderophores, which are low-molecular mass iron chelators that are produced by PGPB, can solubilize iron from minerals or organic compounds under iron limitating conditions to make iron accessible to plants (Indiragandhi et al., 2008). PGPB promote plant growth not only by supplying macro- and micronutrients but also by supplying phytohormones. Indole-3-acetic acid (IAA), which is secreted by PGPB is an important phytohormone and has various effects on plant growth promotion, such as on cell division, elongation and, especially, increasing root development (Ahemad and Kibret, 2014). PGPB can also stimulate plant growth indirectly by suppressing phytopathogens by producing antibiotics, siderophores, and fungal cell wall-lysing enzymes (Ji et al., 2014).

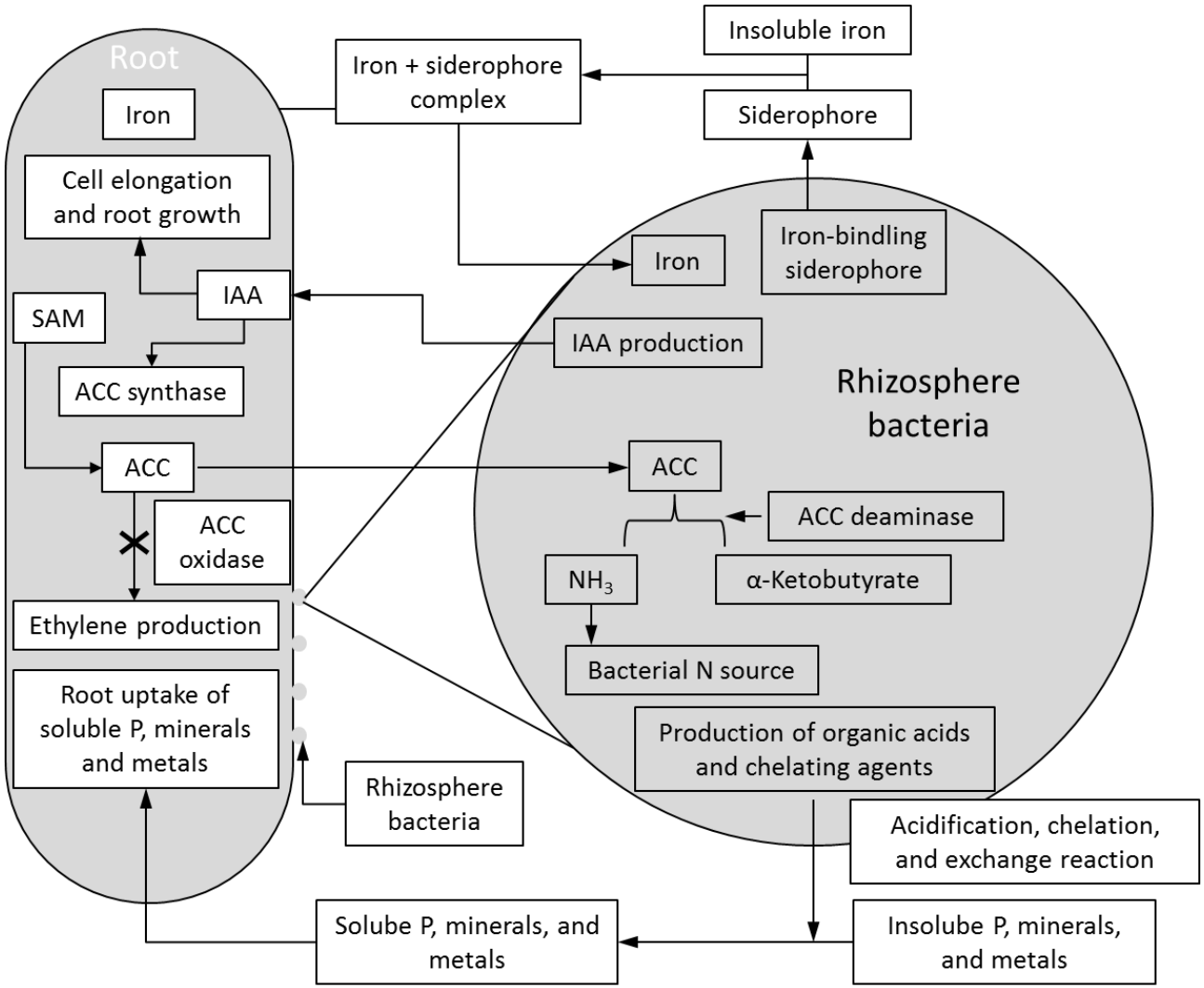

Figure 4: Plant growth promoting mechanisms from bacteria. Source: (Rajkumar et al., 2009)

Substances containing PGPB are defined as biofertilizers which when applied to the soil, increase plant production by supplying nutrients or promoting nutrient uptake by the plant (Vessey, 2003). 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.

6. Biogas plants in Hokkaido

The introduction of biogas plants (BGPs) is a promising measure to produce renewable energy, reduce greenhouse gas emission, recycle organic wastes and provide valuable bio-fertilizer. In Hokkaido, the first biogas plant was built in 1977 in Obihiro University of Agriculture and Veterinary Medicine (Umetsu et al., 2011). Although the initial purpose of biogas plants was for livestock manure treatment, heat and electricity energy produced from generator burning biogas has attracted considerable attention for the building of biogas plants in recent years. Recently, biogas is receiving a great deal of attention as a renewable energy. According to a previous research, there are about 330 biogas plants operating on livestock farms throughout Hokkaido in 2013 (Fig. 5) (Yabe, 2013).

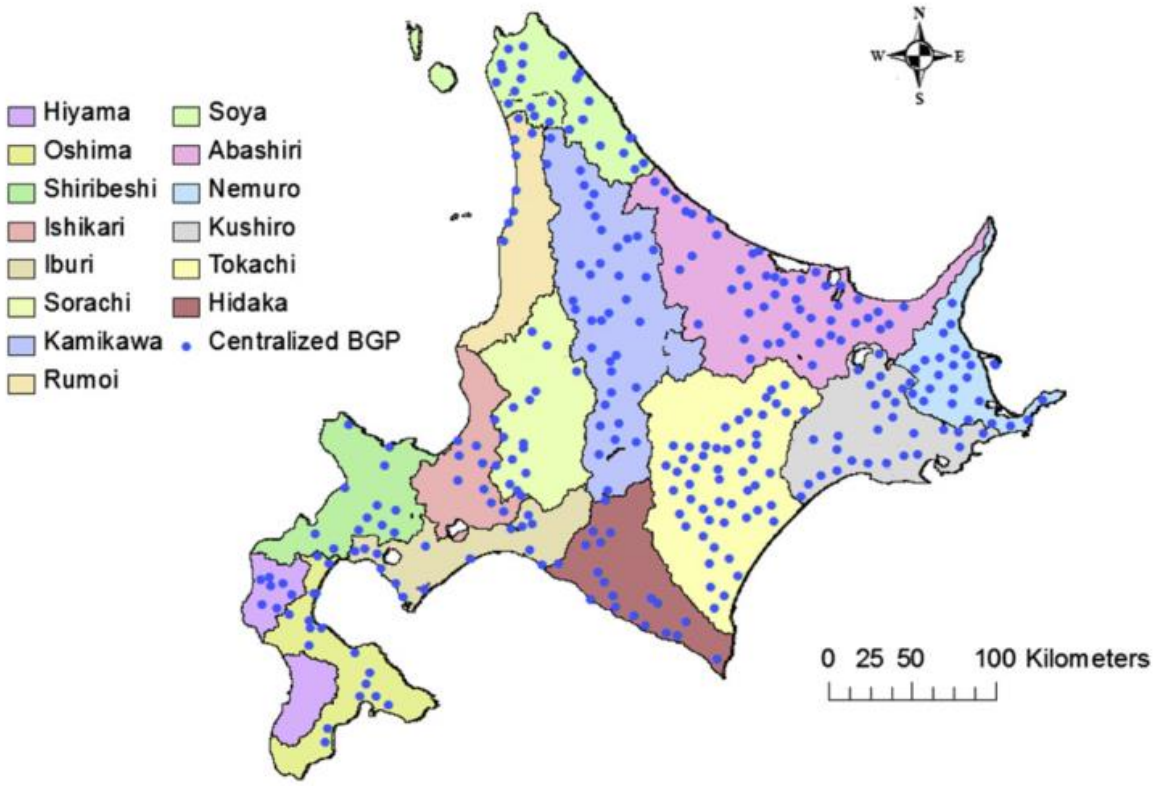

Figure 5: The locations of biogas plants (BGPs) in Hokkaido. Source: (Yabe, 2013)

Objectives and Thesis outlines

The objectives of this PhD thesis were divided into three:

1. To investigate the effects of operating temperature of anaerobic digestion process on fertilizer properties of anaerobic digestate by analyzing plant nutrients content. Furthermore, environmental risks related to pathogenic bacteria and heavy metals were also investigated.

2. To isolate and characterize plant growth promoting bacteria (PGPB) from two types of anaerobic digestate and investigate their growth promotion on common wheat (*Triticum aestivum*) seedlings.

3. To investigate population of plant growth promoting bacteria (PGPB) and pathogenic bacteria in biogas plants in Hokkaido.

Chapter 1 was focused on the effects of operating temperature (mesophilic and thermophilic) on fertilizer properties of anaerobic digestate. Dairy manure was used as feedstock for two laboratory scale continuously anaerobic digesters operated under mesophilic and thermophilic temperatures. Two types of anaerobic digestate were analyzed for the concentrations of the total N and NH_4-N, P (P_2O_5), K (K_2O), Ca (CaO) and Mg (MgO). To evaluate chemical and biological contaminants in anaerobic digestates, the quantities of heavy metals, including Mn, Zn, Cu and Ni, as well as zoonotic bacteria (*Salmonella* and *Campylobacter*) and the *Enterobacteriaceae* genus (*Escherichia coli* and *Enterococcus*) in dairy manure and digestates were also analyzed.

Chapter 2 was focused on the isolation and characterization of plant growth promoting bacteria (PGPB) from anaerobic digestate and their effect on common wheat (*Triticum aestivum*) seedlings growth. *Bacillus* and *Pseudomonas* were isolated from two types of anaerobic digestates, and selected three different plant growth promoting characteristics and antifungal activity to screen 200 bacteria isolated from each digestate. Then bacterial isolates based on plant growth promoting traits were selected and inoculated with common wheat seeds to evaluate their plant growth promoting activity.

Chapter 3 was focused on plant growth promoting bacteria and pathogenic bacteria in anaerobic digestates from two full scale biogas plants in Hokkaido. One is Mikage biogas plant, which was newly operated from spring, 2017. Anaerobic digestate samples were collected from feedstock tank, fermentation tank, sterilization tank and storage tank of Mikage biogas plant. Another one is Shikaoi biogas plant, which has been operated for ten years. Anaerobic digestate samples were only collected from feedstock tank and storage tank of Shikaoi biogas plant.

Chapter 1

Comparative fertilizer properties of digestates from mesophilic and thermophilic anaerobic digestion of dairy manure: Focusing on plant nutrients and environmental risks

Abstract

The fertilizer properties of anaerobic digestate depend on the feedstock and operating conditions of digestion. In this study, the comparative fertilizer properties of mesophilic and thermophilic digestates from dairy manure were evaluated for plant nutrient contents. The contents of pathogenic bacteria and heavy metals were analyzed to determine their environmental risk. The results show that two digestates contained similar plant nutrient contents, while the thermophilic digestate contained higher contents of NH_4^+-N (12.2 g/kg) than 9.8 g/kg in the mesophilic digestate. The reduction rates of pathogenic bacteria were above 90% under thermophilic condition, and the maximum rate was 99.7% for *E. coli*, which were higher than under mesophilic condition (a minimum of 73.2% for *Campylobacter* and maximum of 96.9% for *E. coli*), indicating that thermophilic digestate showed a probable low risk. The lower levels of heavy metals were detected in digestates from dairy manure than those from other feedstocks.

Keywords: Anaerobic digestion; Digestate; Fertilizer property; Environmental risks; Pathogenic bacteria; Heavy metals.

1. Introduction

The appropriate and efficient management of livestock manure is important, as it is a potential hazard to the environment and public health. Anaerobic digestion (AD) provides a promising route to reduce pollution from livestock manure and leads to the formation of biogas, which is a renewable energy source. The digested residue after AD is called digestate and must to be reused to improve the sustainability of the AD process. Recycling digestate as organic fertilizer is considered a suitable use, as it recycles plant nutrients and reduces the consumption of mineral fertilizers (Holm-Nielsen et al., 2009). Furthermore, nutrients are present in inorganic plant-available forms in digestate at a markedly higher level compared to undigested feedstock, due to the mineralization of organic nutrients found in feedstock during AD (Umetsu et al., 2002). The fertilizer properties of digestate highly depend on the composition of feedstock and operating conditions of digestion. In recent years, the fertilizer properties of digestate in various feedstocks have been widely investigated (Abubaker et al., 2012; Alburquerque et al., 2012; Risberg et al., 2017), but little is known about the effect of operating conditions. The AD process can be conducted under different temperatures (mesophilic and thermophilic). Mesophilic digestion requires lower energy cost and is a higher stability process, while thermophilic digestion leads to more rapid digestion and a higher reduction rate of the pathogen. However, little is known about their effects on the fertilizer properties of digestate.

However, agricultural application of anaerobic digestate is not environmentally risk-free, since it may introduce many chemical and biological contaminants into soils (Nkoa, 2014). In recent years, agricultural application of anaerobic digestate has caused public concern due to its increased heavy metals content (Dong et al., 2013). The reason for the increasing concern is that heavy metals are used as feed additives to promote livestock growth, and their contents are known to be increasing in livestock manure, which is used as a substrate for AD (Zhu and Guo, 2014). Therefore, the contents of heavy metals in the digestate should be considered when they are applied. In addition, it is well known that livestock manure contains many pathogenic and non-pathogenic bacteria which may

survive during the AD process and persist in the digestate. Pathogenic bacteria in the digestate are of great concern to the public as they increase the risk of pathogen spread. Pathogenic bacteria are reduced during the AD process, but the reduction rate depends on many factors, such as the fermentation temperature, fermentation time, and initial number of bacterial species in the feedstock (Sahlström, 2003). Therefore, many pathogenic bacteria may still be present in the digestate and cause a health risk for both people and animals.

The main objective of the present study was to compare the fertilizer properties of digestates from mesophilic and thermophilic digestion of dairy manure in terms of their macro and micronutrient contents and environmental risks. Dairy manure was used as feedstock for two laboratory scale continuously anaerobic digesters operated under mesophilic and thermophilic temperatures. The fertilizer properties of two types of anaerobic digestate were estimated by analyzing the concentrations of the total N and NH_4-N, P (P_2O_5), K (K_2O), Ca (CaO) and Mg (MgO). To evaluate chemical and biological contaminants in anaerobic digestates, the quantities of heavy metals, including Mn, Zn, Cu and Ni, as well as zoonotic bacteria (*Salmonella* and *Campylobacter*) and the *Enterobacteriaceae* genus (*Escherichia coli* and *Enterococcus*) in dairy manure and digestates were also analyzed.

2. Materials and Methods

2.1 Anaerobic digestion

Laboratory scale continuously fed anaerobic digestions were performed with stainless-steel cylindrical digesters, with height 29 cm and diameter 29 cm (Yamashiro et al., 2013). The working volume of the digester was 11.2 L. There was a feedstock inlet on the top of the digester and a digestate outlet on the side of the digester. A stirrer was placed inside the digester for mixing feedstock. Dairy manure was collected from the farm of the Obihiro University of Agriculture and Veterinary Medicine and used as feedstock. To begin, the digesters were filled with inoculum and placed in water baths at mesophilic (37°C) and

thermophilic (55°C) temperatures. Mesophilic and thermophilic digesters were fed with 350 g/day and 550 g/day of dairy manure from the feedstock inlet and operated at hydraulic retention times (HRT) of 28.6 and 18.2 days, respectively. Digestates were simultaneously discharged from the outlet by stirring after dairy manure was fed. Dairy manure and digestates were collected during the steady state and analyzed for their pH, total solids (TS) and volatile solids (VS) contents, as well as the concentrations of volatile fatty acids (VFAs), macro- and micronutrients (N, P, K, Ca and Mg) and heavy metals (Mn, Zn, Cu and Ni).

2.2 Detection of pathogenic bacteria

The plate spread method was performed to quantify four pathogenuic bacteria in dairy manure and digestates. Samples were diluted 10-fold with phosphate buffered saline (pH 7.4), and 100 µl of diluent was spread on deoxycholate hydrogen sulfide lactose agar (DHL; Eiken Chemical Co. Ltd., Tokyo, Japan) for *Salmonella* detection, cefoperazone charcoal deoxycholate agar (CCDA; Kanto Chemical, Tokyo, Japan) for *Campylobacter* detection, CHROMagar™ ECC (CHROMagar/Paris, France) for *Escherichia coli* detection and Enterococcosel agar (ECS; Kyokuto Pharmaceuticals Co., Inc., Tokyo, Japan) for *Enterococcus* detection. The incubation time and temperature were controlled according to the specifications. After incubation, typical colonies were counted and calculated as CFU/g dry matter.

2.3 Analytical methods

The daily volume of produced biogas was measured with a wet gas meter. The methane concentration of biogas was determined using a gas chromatograph (GC-14A, Shimadzu, co., Kyoto, Japan) equipped with a thermal conductivity detector (stainless column and Porapak Q packing).

The TS and VS contents of samples were measured according to the standard methods (part 2540G, APHA, 2005). The pH was measured using a Horiba D-55 pH meter. The VFA (formic acid, acetic acid, propionic acid, and butyric acid) concentrations were

determined by HPLC (Shimadzu LC-10AD) with a Shim-Pack SCR-102H column, and the analytical procedures were described by Iwasaki et al., (2013). The concentrations of the total N and NH_4-N, P (P_2O_5), K (K_2O), Ca (CaO) and Mg (MgO) in samples were determined as described by Yamashiro et al. (2013). Heavy metal (Mn, Zn, Cu and Ni) concentrations were determined as described by Dong et al. (2013), and samples were digested with HNO_3/$HClO_4$ (2:1 v/v) at 180°C. After digestion, the samples were filtered with a 0.45-mm filter and used for determination of heavy metal concentrations using inductively coupled plasma-optical emission spectrometry (ICP-OES, PerkinElmer Inc. USA).

2.4 Statistical analysis

Statistical analyses were performed with SAS Statistical Software version 9.4 (SAS Institute Inc., USA). Values with $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1 Anaerobic digestion performance

Anaerobic digestions were operated for approximately 4 months, and their performance was stabilized after the first 2 months, the daily biogas yield, methane concentration and methane yields of last two months are presented in Fig. 1. The mean values of daily biogas production, methane concentration and methane production of mesophilic and thermophilic digesters during the last two months of operation are shown in Table 1. In this study, the thermophilic digester was fed at a higher organic loading rate (OLR) of 4.30 gVS/L/d compared to the 2.73 gVS/L/d OLR of the mesophilic digester and thus showed a higher biogas yield of 1.69 $L/L_{digester}/d$ compared to a biogas yield of 1.22 $L/L_{digester}/d$. The methane concentrations of the biogases produced from both digesters were almost the same at 57.66% and 57.82%, respectively, which are in an acceptable range between 50% and 70%, indicating healthy anaerobic processes (Andriamanohiarisoamanana et al., 2016). Consequently, thermophilic digestion showed a slightly higher value of methane, 0.98 $L/gVS_{digester}/d$, than the 0.71 $L/gVS_{digester}/d$ value of

mesophilic digestion; however, the methane yields per gVS were almost same.

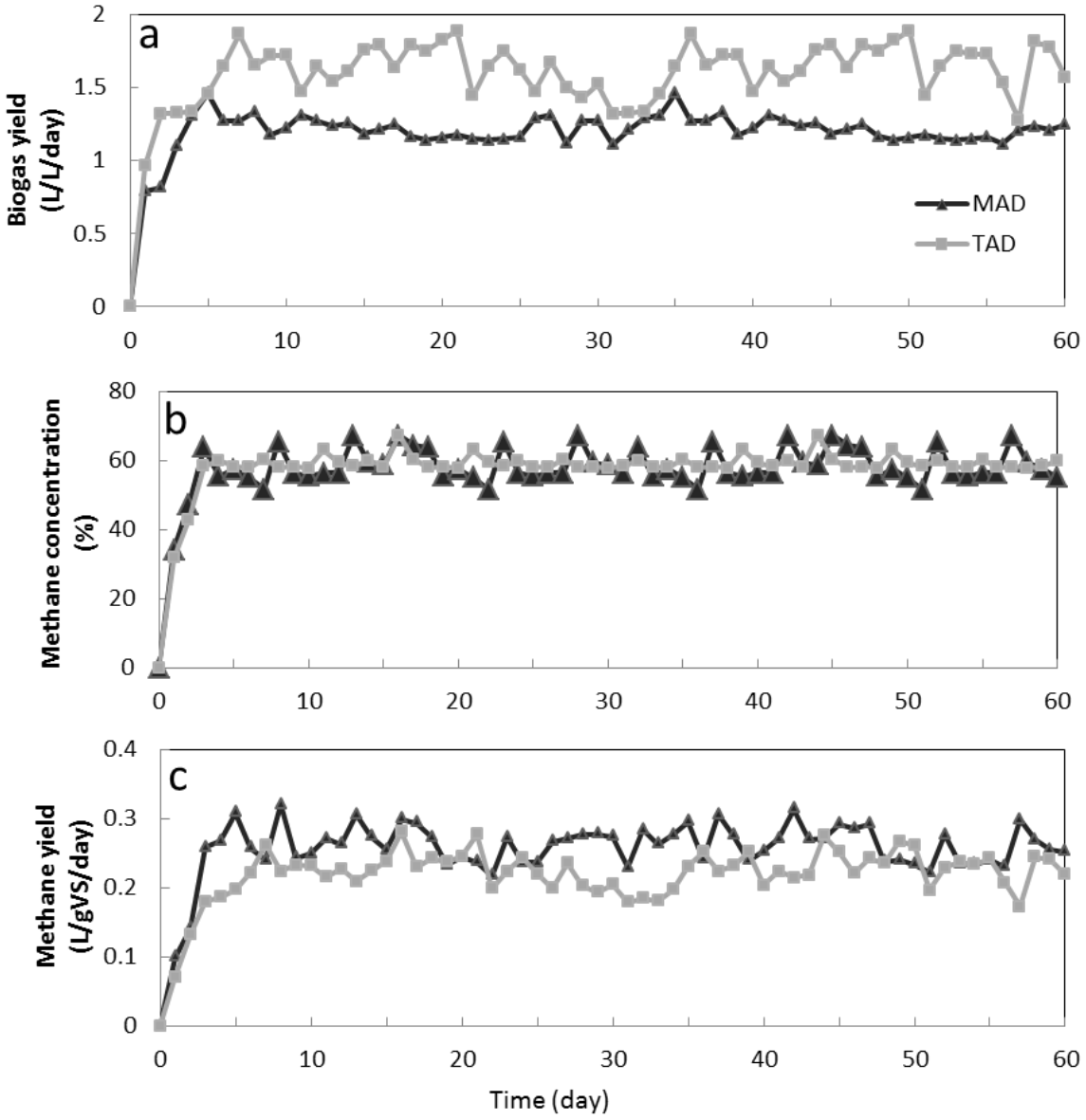

Figure 1: Daily Biogas yields (a), methane concentrations (b) and methane yields (c) of last two months of anaerobic digestions. MAD: Mesophilic anaerobic digestion; TAD: Thermophilic anaerobic digestion

Table 1: Anaerobic digestion performance

	Mesophilic digestion	Thermophilic digestion
Organic loading rate (gVS/L/d)	2.73	4.30
Biogas yield ($L/L_{digester}/d$)	1.22 ±0.07	1.69 ± 0.10
Biogas yield ($L/ gVS_{loaded} /d$)	0.45 ± 0.03	0.37 ± 0.02
Methane concentration (%)	57.66 ± 6.71	57.82 ± 6.12
Methane yield ($L/ L_{digester} /d$)	0.71 ± 0.04	0.98 ± 0.06
Methane yield ($L/gVS_{loaded}/d$)	0.26 ± 0.02	0.23 ± 0.01
VS reduction (%)	46.59	43.18
Methane yield (L/gVSreduction/d)	0.55 ± 0.03	0.53 ± 0.03

Values are present as means with standard deviation.

The TS, VS and pH of dairy manure and digestates are shown in Table 2. TS content was reduced from 10.1% to 6.2 and 6.5%, and VS content was reduced from 8.8% to 4.7 and 5.0% in mesophilic and thermophilic digestates, respectively. The initial pH of the feedstock was 6.3 and increased to 7.7 and 7.6 in the mesophilic and thermophilic digestate, respectively. These results also agreed with those of other researchers (Alburquerque et al., 2012; Yamashiro et al., 2013), the pH of digestate from livestock manure tends towards the alkaline range during AD. These results suggest that the performance of mesophilic and thermophilic anaerobic digestions is similar under specific operating conditions. Both show the same efficiency of methane production and organic solids removal.

Table 2: Total and volatile solid and pH of dairy manure and mesophilic and thermophilic digestates

	Feedstock	Digestates	
	Dairy manure	Mesophilic Digestion	Thermophilic Digestion
TS (Total solid, %)	10.1 ± 0.4	6.2 ± 0.7	6.5 ± 0.5
VS (Volatile solid, %)	8.8 ± 0.7	4.7 ± 0.5	5.0 ± 0.3
pH	6.3 ± 0.2	7.7 ± 0.1	7.6 ± 0.2

Values are present as means with standard deviation.

The concentrations of VFAs in each sample are shown in Fig. 1. The total VFAs in the mesophilic and thermophilic digestates decreased from an initial concentration of 1197.2 mg/L to 38.1 and 103.2 mg/L, respectively, which indicated active consumption of VFAs by methanogens (Riva et al., 2016). Individual volatile fatty acids, especially acetic acid (which was dominant in both dairy manure and the digestates) decreased from 850.6 mg/L to 28.1 and 73.0 mg/L. Propionic acid, the second most common acid, decreased from 212.9 mg/L to 5.2 and 24.8 mg/L in the mesophilic and thermophilic digestates, respectively. The concentration of butyric acid in dairy manure was 127.1 mg/L and decreased to undetectable levels in each digestate. The concentration of each VFA in the thermophilic digestate was higher than in the mesophilic digestate, which was in accordance with Gavala et al. (2003), who reported a high VFA concentration in thermophilic anaerobic digesters due to the relatively high sensitivity of thermophilic anaerobic microorganisms to intermediate compounds.

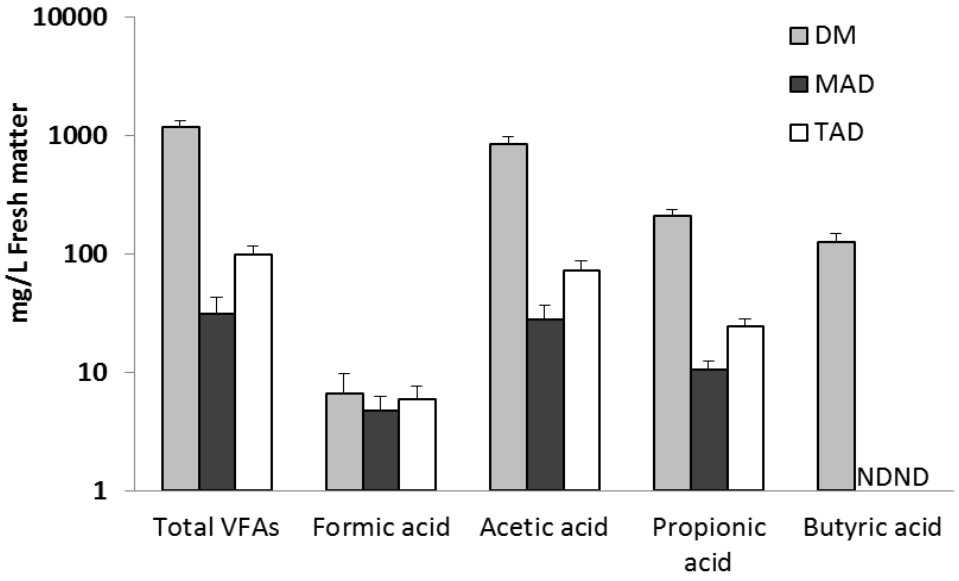

Figure 2: Volatile fatty acids in dairy manure and mesophilic and thermophilic digestates. Values are means with standard deviation. DM: Dairy manure; MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate; ND: Not detected

3.2 Fertilizer properties of mesophilic and thermophilic digestates

Fig. 2 shows the concentrations of macro- and micronutrients in dairy manure and digestates, which indicate fertilizer properties. In this study, the concentrations of N in digestates were detected at higher levels than other macro- and micronutrients at 44.9 and 43.7 g/kg, which was significant higher than the 34.9 g/kg in dairy manure ($p < 0.05$); this range was in accordance with the results of Zirkler et al. (2014), who reported a concentration of 42 to 43 g/kg of N in digestate from cattle slurry. Furthermore, the concentrations of NH_4^+-N increased significantly from 5.3 g/kg to 9.8 g/kg and 12.2 g/kg ($p < 0.05$), likely due to nitrogen fixation and mineralization by methanogens and volatilization of ammonia under anaerobic conditions (Umetsu et al., 2002). Since NH_4^+-N is a more available form for plants, anaerobic digestates from dairy manure had a higher value as a nitrogen fertilizer compared to undigested manure. The concentrations of P, K and Ca in each digestate were of the same level of magnitude (27.0, 29.0 and 28.9 g/kg for

mesophilic digestate and 25.8, 24.9 and 25.8 g/kg for thermophilic digestate), and slightly increased after AD. Similarly, the concentrations of Mg in two digestates increased from 12.4 g/kg to 16.0 and 14.2 g/kg, respectively. The increased macro- and micronutrient concentrations were attributed to weight loss during AD following organic matter conversion and release of biogas (Dong et al., 2013; Micolucci et al., 2016). Therefore, agricultural use of digesates can recycle plant nutrients and reduce the consumption of mineral fertilizers.

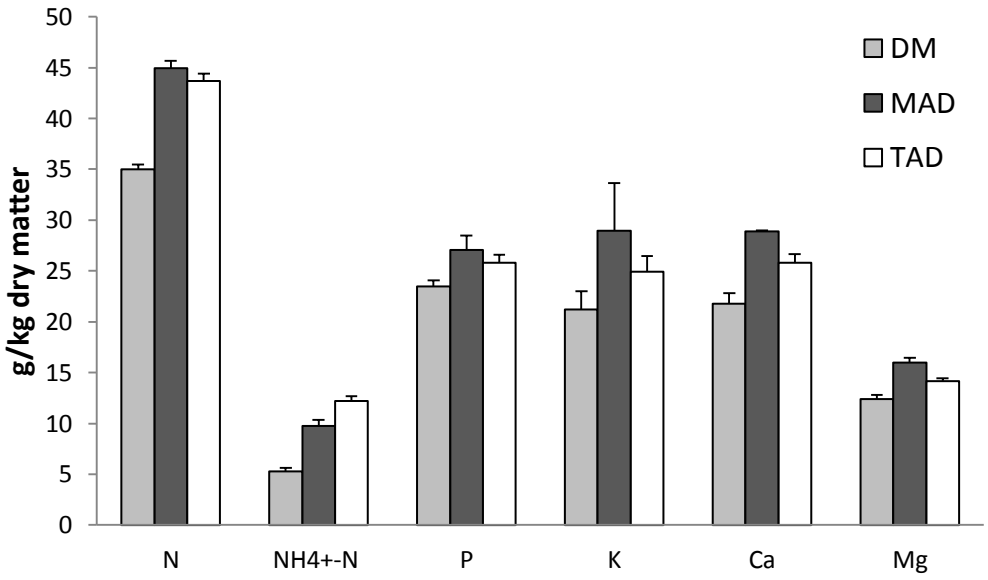

Figure 3: Macro and micronutrients in dairy manure and mesophilic and thermophilic digestates. Values are means with standard deviation. DM: Dairy manure; MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate

3.3 Environmental risk

Livestock manure is considered to contain pathogenic bacteria that might pose a health risk for humans and animals. Pathogenic bacteria may survive during anaerobic digestion and persist in digestates. Digestates from AD must be proven hygienically safe

before they can be applied to soils (Sahlström, 2003). Many researchers have analyzed *E. coli* and *Salmonella* in digestates as hygienic indicators for AD processes (Iwasaki et al., 2011; Micolucci et al., 2016).

In this research, in addition to *E. coli* and *Salmonella*, *Enterococcus* and *Campylobacter* were also detected in dairy manure and digestates (Fig. 3). All four indicator bacteria were more significantly reduced ($p < 0.05$) during the AD process, and the significant difference ($p < 0.05$) in the reduction rate between mesophilic and thermophilic digestion was also found. High temperature was hypothesized to be the factor that led to a high reduction rate for pathogens after thermophilic digestion. Several researchers have also demonstrated that rapid reduction rates for the pathogen were found after thermophilic digestion (Smith et al., 2005; Wagner et al., 2008).

The reduction rate of viable bacteria during AD also depends on the bacterial species and the initial amount of bacteria in the feedstock (Sahlström, 2003). *Salmonella* was reduced by 86.5% and 99.3%, from 5.8 $\log_{10}$CFU/g-dry matter to 5.1 and 3.7 $\log_{10}$CFU/g-dry matter, and *E. coli* was reduced 96.9% and 99.7%, from 6.4 $\log_{10}$CFU/g-dry matter to 4.9 and 3.8 $\log_{10}$CFU/g-dry matter in mesophilic and thermophilic digestates, respectively. Similar results were also reported by Iwasaki et al. (2011), who found that the amount of *E. coli* decreased significantly after mesophilic digestion and that thermophilic digestion eliminated *E. coli* from the feedstock. Goberna et al. (2011) also found that *E. coli* and *Salmonella* were reduced to an undetectable level after AD. *Campylobacter* were reduced by 73.2% and 90.1%, from 4.6 $\log_{10}$CFU/g-dry matter to 4.2 $\log_{10}$CFU/g-dry matter in the mesophilic digestate and 3.7 $\log_{10}$CFU/g-dry matter in the thermophilic digestate, respectively. Similarly, Kearnery et al. (1993) reported that *Campylobacter* could be detected after mesophilic anaerobic digestion under laboratory conditions. *Campylobacter* is one of the major types of bacteria that cause gastroenteritis in human. Alternatively, Stampi et al. (1999) found that *Campylobacter* was sensitive to AD and was eliminated in the digestate. *Enterococcus* was detected at a high level of 7.6 $\log_{10}$CFU/g-dry matter in dairy manure and was reduced by 85.6% and 91.1%, to 6.7 $\log_{10}$CFU/g-dry matter after the mesophilic digestion and 6.5 $\log_{10}$CFU/g-dry matter after the thermophilic digestion,

respectively. Similarly, a rapid reduction rate of *Enterococcus* in thermophilic digestion were reported by Lund et al. (1996), who reported that a four-fold logarithmic reduction of *Enterococcus* was obtained after 300 hours of mesophilic digestion and after 1-2 hours of thermophilic digestion.

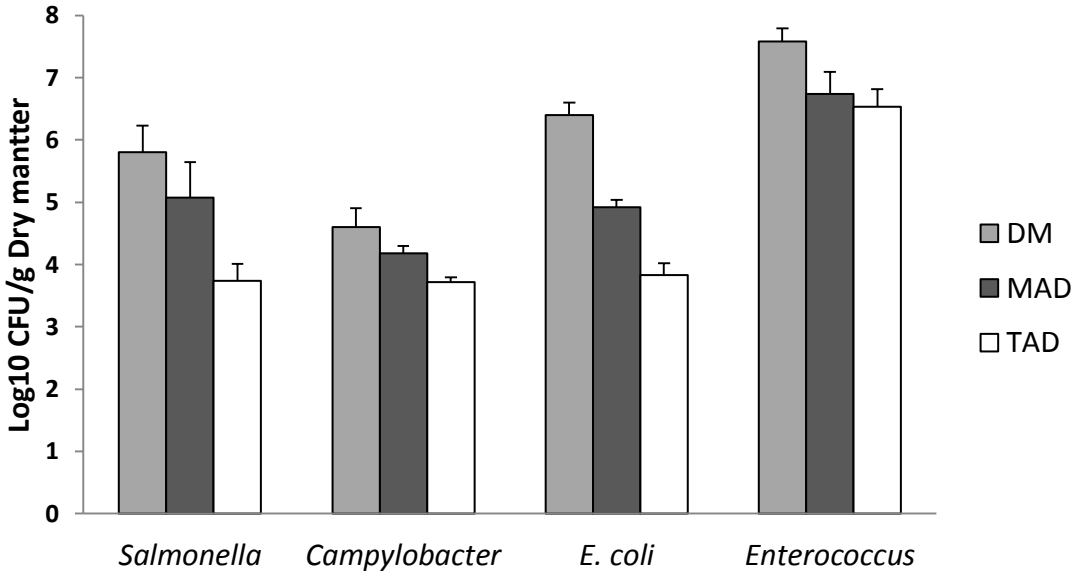

Figure 4: Pathogenic bacterial load in dairy manure and mesophilic and thermophilic digestates. Values are means with standard deviation. CFU: colony forming units; DM: Dairy manure; MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate

In this study, the reduction rates of indicator bacteria were above 90% in the thermophilic digestate, and the maximum rate was 99.7% for *E. coli*, which was higher than in the mesophilic digestate (a minimum of 73.2% for *Campylobacter* and maximum of 96.9% for *E. coli*), indicating that thermophilic digestate showed a probable low risk. However, high residual activity of *Enterococcus* (6.7 and 6.5 $\log_{10}$CFU/g-dry matter) and other indicator bacteria in digestates are considered a possible source of environmental contamination. Therefore, it is important that appropriate management practices are

implemented to minimize the sanitary risks of bacterial transfer to agricultural land from the application of anaerobic digestates.

Heavy metals cause toxicity and other harmful effects not only in plants and soil microorganisms but also humans and animals. In this study, the contents of Mn, Cu, Zn and Ni in dairy manure and digestates were analyzed (Table 3), and the order of total metal content in the digestates was Zn > Mn > Ni > Cu. The concentrations of these heavy metals in dry matter were typically higher in digestates than in dairy manure, in accordance with the results of Dong et al. (2013) and Micolucci et al. (2016), due to weight loss in AD process following organic matter conversion and release of biogas (Dong et al., 2013; Micolucci et al., 2016). The increase in total concentrations of heavy metals in digestates is likely to show an increased risk to the environment. However, the heavy metal concentrations of digestates in this study were lower than in other feedstock.

Table 3 also compares heavy metal concentrations in anaerobic digestates from various feedstocks. The concentrations of Cu in anaerobic digestates from dairy manure were 44.4 and 43.5 mg/kg, which was less than those from anaerobic digestion of sewage sludge (275.9 mg/kg) and biowaste (68.1 and 52.5 mg/kg) (biowaste: fruits, vegetables and kitchen waste). The concentrations of Zn in digestates of dairy manure were 364.4 and 325.2 mg/kg, which were also much less than digestates of sewage sludge (2126.8 mg/kg) but higher than from biowaste (155.0 and 129.0 mg/kg). For Mn, the concentrations (291.2 and 267.8 mg/kg) were much less than in a digestate from pig slurry (1900.9 mg/kg) (Zhu and Guo, 2014). In contrast, only the concentrations of Ni in digestates were detected at a higher level (183.7 and 162.5 mg/kg) than in both sewage sludge (157.1 mg/kg) and biowaste (42.1 and 27.0 mg/kg). These differences indicate that the level of heavy metals in digestates is highly dependent on their concentrations in the feedstock. Therefore, it is imperative to remove these heavy metals before the application of anaerobic digestate when the feedstock used, such as sewage sludge, contains high contents of heavy metal.

Table 3: Heavy metals concentrations in dairy manure and mesophilic and thermophilic digestates

		Heavy metal concentrations (mg/kg dry matter)			
	Feedstock type	Feedstock	MAD[a]	TAD	Reference
Cu	Dairy manure	31.4 ± 2.7 [b]	44.4 ± 1.3	43.5 ± 1.7	This study
	Sewage sludge	181.7 ± 3.5	275.9 ± 10.5	ND[d]	Dong et al. (2013)
	Biowaste[c]	47.0 ± 5.0	68.1 ± 3.2	52.5 ± 7.8	Micolucci et al. (2016)
Zn	Dairy manure	280.1 ± 25.6	364.4 ± 2.6	325.2 ± 24.7	This study
	Sewage sludge	1453.9 ± 19.1	2126.8 ± 21.6	ND	Dong et al. (2013)
	Biowaste	112.0 ± 28.0	155.0 ± 13.0	129.0 ± 32.0	Micolucci et al. (2016)
Ni	Dairy manure	120.3 ± 15.8	183.7 ± 0.7	162.5 ± 10.3	This study
	Sewage sludge	114.8 ± 3.3	157.1 ± 5.3	ND	Dong et al. (2013)
	Biowaste	43.7 ± 3.0	42.1 ± 1.6	27.0 ± 0.5	Micolucci et al. (2016)
Mn	Dairy manure	207.5 ±10.7	291.2 ± 6.8	267.8 ± 7.0	This study
	Pig slurry	ND	1900.9 ± 38.1	ND	Zhu and Guo, (2014)

[a] MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate.

[b] Values are present as means with standard deviation.

[c] Biowaste: Fruits, vegetables and kitchen waste.

[d] ND: Not detected.

4. Conclusion

The comparative fertilizer properties of mesophilic and thermophilic digestates of dairy manure have been evaluated regarding plant nutrient and environmental risks. The results showed that operating temperature of anaerobic digestion is the major determinant factor that affecting the fertilizer properties of anaerobic digestate. High temperature leads to the high contents of inorganic plant-available nutrients and high reduction rate of pathogenic bacteria after anaerobic digestion process. The results also showed that digestates from dairy manure contained lower levels of heavy metals than those from other feedstocks.

Chapter 2

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

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 characteristics 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 6.3 and 4.8 CFU g^{-1} dry matter in mesophilic digestate, while were 5.8 and 4.7 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 characteristics 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*).

1. 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*).

2. Materials and Methods

2.1 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. Dairy manure and digestate samples were immediately kept at 4°C and isolation of bacteria was done within 24 h.

2.2 Isolation of *Bacillus* and *Pseudomonas*

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 Plant growth promoting characteristics 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 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): $PGI = (1 – R/Rc) \times 100\%$, 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 *Bacillus* and *Pseudomonas*

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).

3. Results and Discussion

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

The bacterial concentration of *Bacillus* and *Pseudomonas* in dairy manure and digestates are shown in Fig. 1. Generally, microorganisms are thought to be inactivated during AD due to temperature, retention time, and VFA concentration in combination with pH (Sahlström, 2003; Smith et al., 2005; Wagner et al., 2008). However, this study showed that *Bacillus* in dairy manure increased significantly ($p < 0.05$), by 5.8-fold and 1.1-fold,

from 5.5 log_{10}CFU/g-dry matter to 6.3 and 5.8 log_{10}CFU/g-dry matter under mesophilic and thermophilic conditions, respectively. Similarly, some studies also found that the number of spore-formers, such as *Bacillus* spp., was not reduced after AD (Bagge et al., 2005; Sahlström et al., 2004), which may be attributed to spores being more robust and resistant to elevated temperatures (Kumar et al., 2012). In this study, it appeared that the suitable temperatures and available nutrients in digesters stimulated the growth of *Bacillus*. However, it is not possible to affirm from this study that the observed phenomenon was a result of suitable temperatures and available nutrients. Further research is recommended to ascertain the role of temperature and nutrients on the growth of *Bacillus* in the anaerobic digester. In contrast, *Pseudomonas* decreased significantly ($p < 0.05$) by 39.8% and 51.3%, from 5.1 log_{10}CFU/g-dry matter to 4.8 and 4.7 log_{10}CFU/g-dry matter, respectively. Furthermore, the quantities of both *Bacillus* and *Pseudomonas* in the digestates were significantly different ($p < 0.05$).

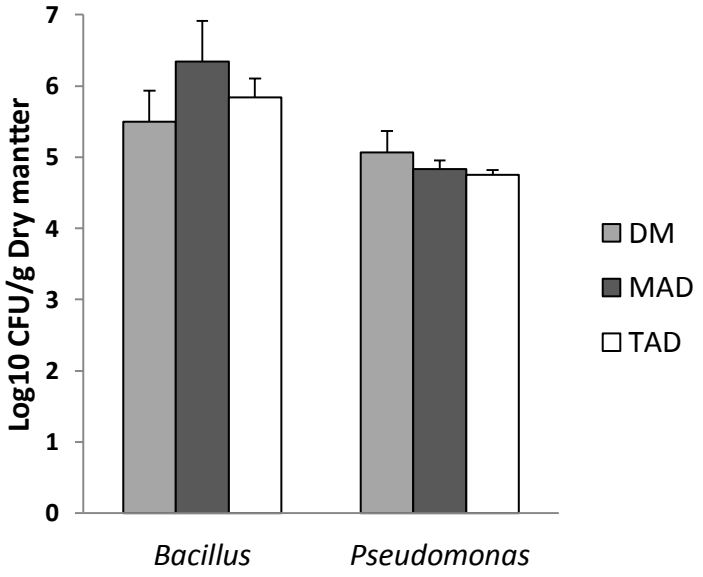

Figure 1: Cultivable population of *Bacillus* and *Pseudomonas* in dairy manure and mesophilic and thermophilic digestates. Values are means with standard deviation. CFU: colony forming units; DM: Dairy manure; MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate

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 characteristics and antifungal activity from anaerobic digestates

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

[a] MAD: Mesophilic anaerobic digestate; TAD: Thermophilic anaerobic digestate.

[b] IAA: Indole-3 acetic acid.

3.2 Plant growth promoting characteristics and antifungal activity

Bacterial isolates that were found to be positive in one or more plant growth promoting activities are presented in Table 2 and 3. Twelve (MAD-01 to 12) *Bacillus* isolates and 13 (MAD-13 to 25) *Pseudomonas* isolates from the mesophilic digestate showed plant growth promoting characteristics (Table 3). All 12 *Bacillus* isolates that showed siderophore production but no or limited IAA production (under 4μg ml^{-1}) was identified as *Bacillus subtilis*. All 13 *Pseudomonas* isolates that produced siderophores and a high level of IAA (11.6 to 55.6 μg ml^{-1}) were identified as fluorescent pseudomonads. Among these isolates, only MAD-21 showed phosphate solubilization and was identified as *Pseudomonas putida* (Fig. 2). In the thermophilic digestate, only five *Bacillus* isolates (TAD-01 to 05) and seven *Pseudomonas* (TAD-06 to 12) isolates showed plant growth promoting characteristics (Table 4). Four *Bacillus* isolates (TAD-01 to 04) were *Bacillus subtilis* and showed siderophore production and little or no IAA production; these exhibited the same characteristics as the *Bacillus* isolates from the mesophilic digestate. Another *Bacillus* isolate, TAD-05, was identified as *Bacillus licheniformis* and showed a high level of IAA production (35.1 μg ml^{-1}) but no siderophore production. Six *Pseudomonas* isolates (TAD-06 to 11) showed siderophore production, but their IAA production differed widely (4.2 to 33.1 μg ml^{-1}). Among all *Pseudomonas* isolates, TAD-12, which showed a maximum IAA production of 75.2 μg ml^{-1}, was found to be *Pseudomonas aeruginosa*.

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, 12 *Bacillus* isolates from a mesophilic digestate showed antifungal activity from 56.1% to 75.3%, while five *Bacillus* isolates from a thermophilic digestate showed antifungal activity from 43.5% to 70.6% (Table 2 and 3). In contrast, no *Pseudomonas* isolates showed antifungal activity (Fig. 3). The antifungal activity of the isolates was not correlated with production of siderophores, which was in accordance with results of Grobelak et al. (2015), which could be due to the competition for space and nutrients and secretion of antifungal compounds between *Bacillus* isolates and

fungal strains caused the antifungal activity (Yang et al., 2015).

Figure 2: The colony of MAD-21 *Pseudomonas putida* on CAS and Pikovskaya's agar plate. Left: Orange halo on CAS plate indicate production of siderophore; Right: Clear halo around the colony indicate phosphate solubilization

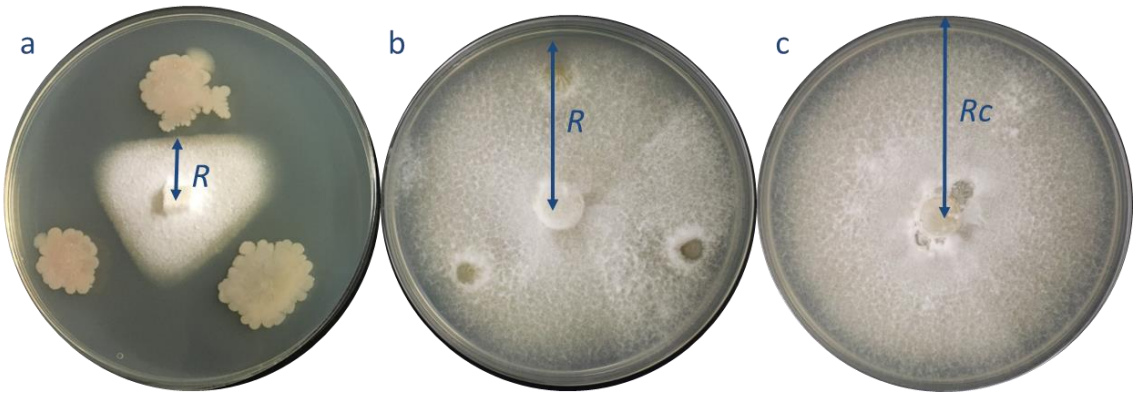

Figure 3: Antifungal activities of bacteria against *Fusarium nivale* (a) MAD-07, (b) TAD-06, (c) control. The percent of growth inhibition (PGI) = (1 − R/Rc) × 100; R = radius of fungal mycelia in bacteria-inoculated plate; Rc = radius of fungal mycelia in control plate

Table 2: Plant growth promoting characteristics of bacterial isolates from mesophilic digestate

Bacterial isolate no. [a]	Phosphates solubilization[b]	Siderophores production [c]	IAA production (μg ml^{-1}) [d]	Antifungal activity(PGI%)[e]	Identification[f]
MAD-01	-	+	-	72.2	*Bacillus subtilis*
MAD-02	-	+	-	75.3	*Bacillus subtilis*
MAD-03	-	+	-	63.9	*Bacillus subtilis*
MAD-04	-	+	-	60.8	*Bacillus subtilis*
MAD-05	-	+	3.2	62.8	*Bacillus subtilis*
MAD-06	-	+	-	56.1	*Bacillus subtilis*
MAD-07	-	+	2.8	61.6	*Bacillus subtilis*
MAD-08	-	+	3.9	56.1	*Bacillus subtilis*
MAD-09	-	+	3.2	63.5	*Bacillus subtilis*
MAD-10	-	+	-	62.4	*Bacillus subtilis*
MAD-11	-	+	-	56.9	*Bacillus subtilis*
MAD-12	-	+	3.0	65.1	*Bacillus subtilis*
MAD-13	-	+	26.3	-	fluorescent pseudomonads
MAD-14	-	+	23.4	-	fluorescent pseudomonads
MAD-15	-	+	21.1	-	fluorescent pseudomonads
MAD-16	-	+	30.1	-	fluorescent pseudomonads
MAD-17	-	+	51.2	-	fluorescent pseudomonads
MAD-18	-	+	15.8	-	fluorescent pseudomonads
MAD-19	-	+	32.7	-	fluorescent pseudomonads
MAD-20	-	+	47.0	-	fluorescent pseudomonads
MAD-21	+	+	55.6	-	*Pseudomonas putida*
MAD-22	-	+	17.6	-	fluorescent pseudomonads
MAD-23	-	+	27.3	-	fluorescent pseudomonads
MAD-24	-	+	38.7	-	fluorescent pseudomonads
MAD-25	-	+	11.6	-	fluorescent pseudomonads

[a] MAD: Mesophilic anaerobic digestate.

[b] Phosphate solubization (+); non phosphate solubization (-).

[c] Siderophores production (+);non siderophores production (-).

[d] IAA: Indole-3acetic acid; values are expressed as means; non IAA production (-).

[e] PGI: percent of growth inhibition; values are expressed as means; non growth inhibition (-).

[f] Identified by gram stain, microscopic morphology, oxygen preference and MALDI TOF/MS.

Table 3: Plant growth promoting characteristics of bacterial isolates from thermophilic digestate

Bacterial isolate no. [a]	Phosphates solubilization[b]	Siderophores production [c]	IAA production (μg ml^{-1}) [d]	Antifungal activity(PGI%)[e]	Identification[f]
TAD-01	-	+	3.2	70.2	*Bacillus subtilis*
TAD-02	-	+	-	63.1	*Bacillus subtilis*
TAD-03	-	+	-	52.2	*Bacillus subtilis*
TAD-04	-	+	2.9	70.6	*Bacillus subtilis*
TAD-05	-	-	35.1	43.5	*Bacillus licheniformis*
TAD-06	-	+	26.7	-	fluorescent pseudomonads
TAD-07	-	+	6.3	-	fluorescent pseudomonads
TAD-08	-	+	5.8	-	fluorescent pseudomonads
TAD-09	-	+	33.1	-	fluorescent pseudomonads
TAD-10	-	+	4.2	-	fluorescent pseudomonads
TAD-11	-	+	18.9	-	*Pseudomonas* spp.
TAD-12	-	-	75.2	-	*Pseudomonas aeruginosa*

[a] TAD: Thermophilic anaerobic digestate.

[b] Phosphate solubization (+); non phosphate solubization (-).

[c] Siderophores production (+);non siderophores production (-).

[d] IAA: Indole-3acetic acid; values are expressed as means; non IAA production (-).

[e] PGI: percent of growth inhibition; values are expressed as means; non growth inhibition (-).

[f] Identified by gram stain, microscopic morphology, oxygen preference and MALDI TOF/MS.

3.3 The fertilizer properties of anaerobic digestate related to PGPB

Phosphorus is considered one of the most important nutrients for plant growth. However, a large proportion of phosphorus in soil is present in insoluble forms and is consequently not available for plant nutrition. Application of a digestate is thought to affect phosphorous availability in the soil either directly by adding inorganic phosphorous or indirectly by influencing soil microbial activity (Insam et al., 2015). In this study, phosphate solubilizing *Pseudomonas putida* was detected in the mesophilic digestate. Kaur and Sudhakara Reddy (2014) found that inoculation of phosphate solubilizing bacteria significantly increased plant phosphorous uptake and available phosphorous in soil samples. Therefore, application of a mesophilic digestate may increase phosphorous availability in the soil by directly introducing phosphate solubilizing bacteria to agricultural soil.

Iron (Fe) is an essential micronutrient for both plants and microorganisms. In soil, iron mainly presents as Fe^{3+}, which is likely to form insoluble hydroxides and oxyhydroxides, making it inaccessible to both plants and microorganisms (Ahemad and Kibret, 2014). PGPB produce siderophores and make iron accessible to plants by solubilizing iron under iron-limiting conditions (Indiragandhi et al., 2008). In this study, 25 bacterial isolates from mesophilic digestate and 10 bacterial isolates from thermophilic digestate showed siderophores production, and therefore, the application of digestate might change iron conditions in soils.

In this study, fluorescent pseudomonads were detected in both mesophilic and thermophilic digestates and showed high IAA production ranging from 4.2 to 51.2 μg ml^{-1}. *Pseudomonas aeruginosa* (TAD-12) was found in a thermophilic digestate and showed the highest IAA production (75.2 μg ml^{-1}). Li et al. (Li et al., 2016) found that liquid digestate contained a high concentration of IAA (21.2-22.0 mg ml^{-1}), but the reason has not been elucidated. According to this study, PGPB in digestate may contribute to IAA content in digestate. IAA is a phytohormone that is involved in root initiation, cell division and cell enlargement. It is well known that inoculation with IAA-producing bacteria increases plant

growth by promoting root growth and length (Vessey, 2003). Therefore, application of digestate with IAA-producing PGPB could increase root development and increase growth by supplying this phytohormone to plants.

Some reports confirm a biocontrol effect of digestate on plant diseases. Kupper et al. (Kupper et al., 2006) reported the biocontrol of anaerobic digestate against citrus black spot disease caused by *Phyllosticta citricarpa*. However, there is limited research on the mechanisms of plant fungal pathogen inhibition by anaerobic digestate. In this study, 12 *Bacillus* isolates from a mesophilic digestate showed antifungal activity from 56.1% to 75.3%, while five *Bacillus* isolates from a thermophilic digestate showed antifungal activity from 43.5% to 70.6% (Table 3 and 4). *Bacillus* species have been widely reported to have antifungal activity against many phytopathogens, such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Xanthomonas campestris*, *Macrophomina phaseolina*, and *Sclerotinia sclerotiorum* (Ji et al., 2014; Kumar et al., 2012; Liu et al., 2016). Therefore, application of a digestate might protect plants from phytopathogens or decrease disease symptoms, due to the presence of *Bacillus* species.

As described above, *Bacillus* and *Pseudomonas* species isolated from digestates showed various plant growth promoting characteristics and antifungal activity. These bacteria may be able to colonize the rhizosphere with digestate application and may increase the availability of nutrients and decrease disease symptoms, which make digestate an effective biofertilizer. Biofertilizer is not only suitable for use as a soil conditioner and fertilizer, but can also suppress soil-borne phytopathogens (Alfa et al., 2014; Owamah et al., 2014). Therefore, in addition to plant nutrients, the PGPB content in the digestate should be taken into account when considering the fertilizer properties of the digestate. In this study, 25 tested bacterial isolates from the mesophilic digestate showed plant growth promoting characteristics, which was significantly higher than 12 isolates from the thermophilic digestate according to the binomial distribution test. These results indicated that temperature affected PGPB content in anaerobic digestates.

3.4 Bacterial isolates selected for plant growth promoting assay

For plant growth promoting assay, 6 bacterial isolates (MAD-05: *Bacillus subtilis;* MAD-17: Fluorescent pseudomonads*;* MAD-21: *Pseudomonas putida;* TAD-05: *Bacillus licheniformis;* TAD-11: *Pseudomonas spp.;* TAD-21: *Pseudomonas aeruginosa*) were selected for plant growth promoting assay. 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).

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).

3.5 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. 2 and 3). Stem length of the seedlings inoculated with bacterial isolates (Fig. 3A) 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. 3A). The bacterial isolates inoculation further increased biomass of seedlings stem and root (Fig. 3B). 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 ($p < 0.05$).

Figure 4: 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.

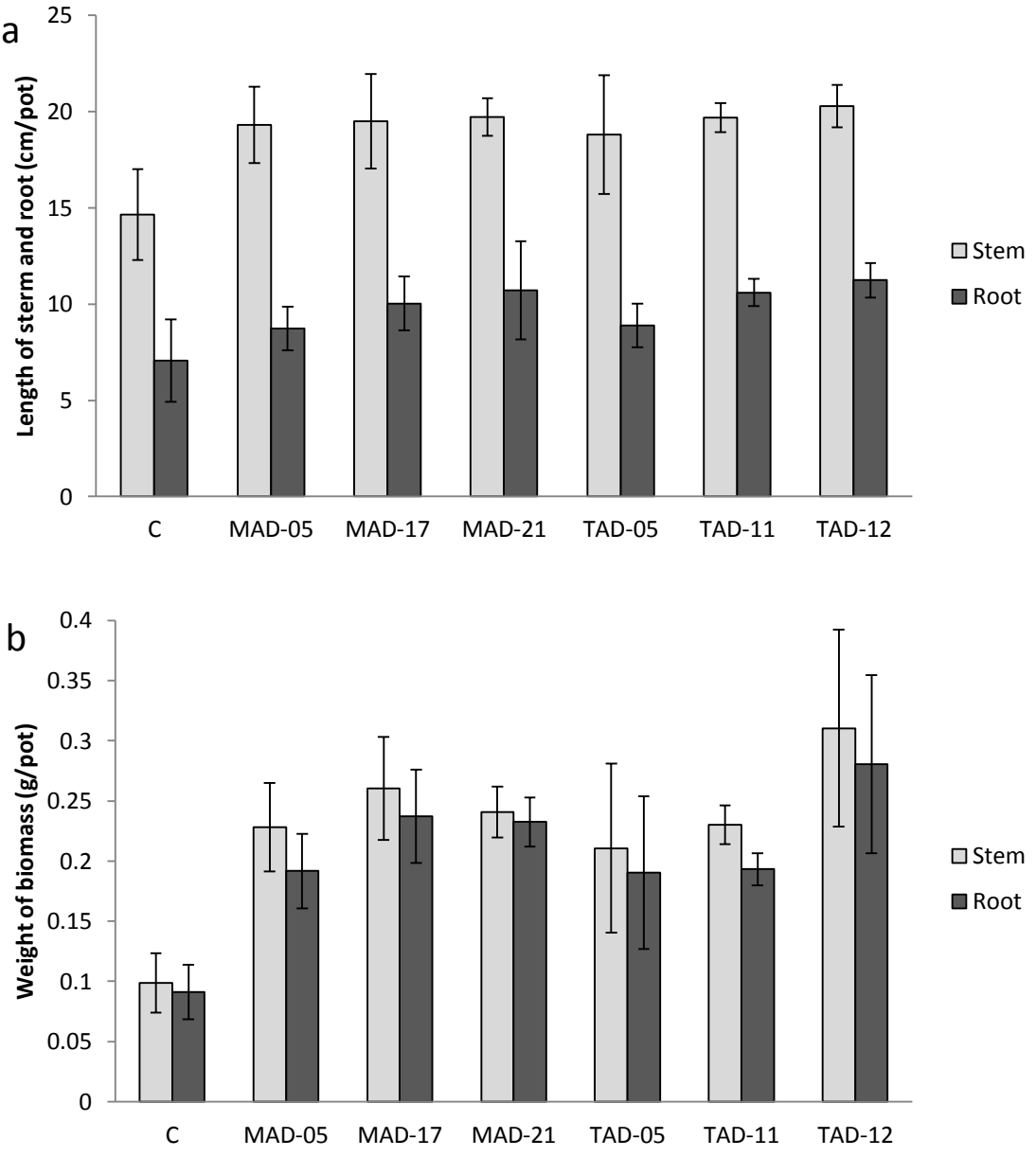

Figure 5: Stem and root length (a) and weight (b) of common wheat seedlings inoculated with bacterial isolates in plant growth promoting assay. C: Untreated control; MAD-05: *Bacillus subtilis*; MAD-17: Fluorescent pseudomonads; MAD-21: *Pseudomonas putida*; TAD-05: *Bacillus licheniformis*; TAD-11: *Pseudomonas* spp.; TAD-21: *Pseudomonas aeruginosa*

4. 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 characteristics 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.

Chapter 3

Investigation of plant growth promoting bacteria (PGPB) and pathogenic bacteria in biogas plants (BGPs) in Hokkaido

Abstract

The introduction of biogas plants (BGPs) is a promoting measure to recycle organic wastes, produce renewable energy and reduce greenhouse gas. Application of anaerobic digestate from BGPs as a fertilizer for agriculture also reduces the consumption of mineral fertilizers. In this chapter, the fate of plant growth promoting bacteria and pathogenic bacteria in two full scale biogas plants in Hokkaido were investigated. One is Mikage biogas plant, which was newly operated from spring, 2017. Slurry samples were collected from feedstock tank, fermentation tank, sterilization tank and storage tank. Another one is Shikaoi biogas plant, which has been operated for ten years. Slurry samples were only collected from feedstock tank and storage tank of Shikaoi biogas plant. The results showed that treatment at BGPs inactivated pathogenic bacteria in feedstock, which confirm the security of digestion application. However, *Campylobacter* residue is considered a possible source of environmental contamination. The presence of *Bacillus* also makes anaerobic digestate a potential bio-fertilizer.

Keywords: Biogas plants (BGPs); Anaerobic digestion; Plant growth promoting bacteria (PGPB); Pathogenic bacteria.

1. Introduction

When untreated or inappropriately managed, livestock manure becomes a potential hazard source to the environment and public health (Holm-Nielsen et al., 2009; Yamashiro et al., 2013). If treated properly, livestock manure however, can be a valuable biomass for renewable energy production and a source of bio-fertilizer for agriculture. Biogas plants (BGPs) provide an eco-friendly treatment for various organic wastes including livestock with anaerobic microorganisms in the absence of oxygen and produce a biogas consisting of methane (CH_4) and carbon dioxide (CO_2), which is called anaerobic digestion (AD) process. This biogas can be used directly for heat and electricity generation or upgraded to high-quality bio-methane as fuel for vehicle (Jiang et al., 2011). Recently, BGPs is receiving a great of deal of attention as a measure to recycle organic wastes, produce renewable energy, reduce greenhouse gas (GHG) (Umetsu et al., 2011; Yabe, 2013).

Hokkaido is the most northern island of Japan with a total of 847,000 cows, which is half the total number in Japan (MAFF, Ministry of Agriculture, Forestry and Fisheries, 2008). Moreover, the scale of dairy farm is much larger than in other regions, which making Hokkaido an appropriate region to introduce BGPs. With the development of BGPS in Hokkaido, the amount of anaerobic digestate, the digested residue after AD process, also increased sharply. The sustainability of farm-scale BGPs depends highly on the appropriate disposal of anaerobic digestate produced with biogas (Alburquerque et al., 2012). Recycling digestate as an organic fertilizer is considered the most suitable utilization of digestate, as it recycles plant nutrients and reduces the consumption of mineral fertilizers (Holm-Nielsen et al., 2009).

Livestock manure is considered to contain pathogenic bacteria that might pose a health risk for humans and animals. Pathogenic bacteria may survive during anaerobic digestion and persist in digestates. Digestates from AD must be proven hygienically safe before they can be applied to agricultural lands (Sahlström, 2003). However, the regulation concerning the hygienic standard of BGPs digesate is limited in Japan (Iwasaki et al., 2011). Pathogenic bacteria are reduced during the AD process, but the reduction rate

depends on many factors, such as the fermentation temperature, fermentation time, and initial number of bacterial species in the feedstock (Smith et al., 2005). Previous researches have investigated the survival of pathogenic bacteria during AD process in lab-scale (Alfa et al., 2014; Micolucci et al., 2016; Owamah et al., 2014). However, there is limited data on survival of pathogenic bacteria in full-scale BGPs. In addition, plant growth promoting bacteria (PGPB), such as *Bacillus* and *Pseudomonas* were isolated and showed growth promotion on common wheat in Chapter 2. The presence of these bacteria makes the anaerobic digestate a potential bio-fertilizer. Therefore, it is necessary to investigate the fate of *Bacillus* and *Pseudomonas* in full-scale BGPs.

The objective of this chapter was to investigate plant growth promoting bacteria (PGPB) and pathogenic bacteria in two full-scale biogas plants (BGPs) in Mikage and Shikaoi, Hokkaido. Anaerobic digestate samples were collected from feedstock tank, fermentation tank, sterilization tank and storage tank of Mikage biogas plant. Another one is Shikaoi biogas plant, which has been operated for ten years. Anaerobic digestate samples were only collected from feedstock tank and storage tank of Shikaoi biogas plant. Plant growth promoting *Bacillus* and *Pseudomonas*, zoonotic bacteria (*Salmonella* and *Campylobacter*) and the genera of *Enterobacteriaceae* (*Escherichia coli* and *Enterococcus*) were detected from samples.

2. Materials and Methods

2.1 Anaerobic digestate samples collection

Anaerobic digestate samples were collected from two biogas plants located in Mikage town and Shikaoi town, Tokachi, Hokkaido. Classifications of two biogas plants and operating conditions in this study are presented in Table 1. Slurry samples from Mikage biogas plant were collected from feedstock tank, fermentation tank, sterilization tank and storage tank. While slurry samples from Shikaoi biogas plant were collected from feedstock tank and storage tank. All samples were immediately kept at 4°C, and analyses and bacteria detection were conducted within 24 h.

2.2 Detection of plant growth promoting bacteria (PGPB)

Bacillus and *Pseudomonas* in digestate samples were quantified 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) and Difco™ Cetrimide Agar Base (Becton, Dickinson and Company, Sparks, MD, USA) plates for quantification of *Bacillus* and *Pseudomonas*, respectively. The incubation time and temperature were controlled according to the specifications. After incubation, typical colonies were counted and calculated as colony forming units per gram of dry matter (CFU/g dry matter).

2.3 Detection of pathogenic bacteria

The plate spread method was performed to quantify pathogenic bacteria in digestate samples. Samples were diluted 10-fold with phosphate buffered saline (pH 7.4), and 100 µl of diluent was spread on deoxycholate hydrogen sulfide lactose agar (DHL; Eiken Chemical Co. Ltd., Tokyo, Japan) for *Salmonella* detection, cefaperazone charcoal deoxycholate agar (CCDA; Kanto Chemical, Tokyo, Japan) for *Campylobacter* detection, CHROMagar™ ECC (CHROMagar/Paris, France) for *Escherichia coli* detection and Enterococcosel agar (ECS; Kyokuto Pharmaceuticals Co., Inc., Tokyo, Japan) for *Enterococcus* detection. The incubation time and temperature were controlled according to the specifications. After incubation, typical colonies were counted and calculated as CFU/g dry matter.

2.4 Analytical methods

Total solids (TS) were determined by drying samples in a fan-assisted oven at 105°C for 24 h, and TS contents were calculated from the differences in weights of samples. Thereafter, volatile solids (VS) were determined by combusting dried samples at 550°C for 4 h. The pH was measured using a Horiba D-55 pH meter. The VFAs (acetic acid, propionic acid, and butyric acid) concentrations were determined by high performance liquid chromatography (HPLC, LC-10AD, Shimadzu Co., Japan) with a Shim-Pack SCR-102H

column. 3 g sample was pre-treated with 6 mL of 10% tungsten acid and 6 mL of 7% sulfuric acid. The mixture was homogenized for 5 min, and then centrifuged at 10,000 g for 20 min. The supernatant of sample was collected and analyzed by HPLC. 5 mM p-toluene sulfonic acid was used as mobile phase at flow rate of 0.8 mL/min at 45 °C. Buffer phase was a mixture of 5 mM p-toluene sulfonic acid, 20 mM Bis-Tris and 100 μM EDTA (Iwasaki et al., 2013).

3. Results and Discussion

3.1 Classifications and operating conditions of the biogas plants

The classifications and operating conditions of the two biogas plants are presented in Table 1. Mikage biogas plant was built from 2015 and operated in spring 2017. Shikaoi biogas plant has been operated for 10 years from 2007. Both two biogas plants are operated at mesophilic temperature, which can be attributed to low energy cost and high process stability of mesophilic anaerobic digestion (Gavala et al., 2003). Shikaoi biogas plant is treating dairy manure combined with food waste, which is also called anaerobic co-digestion. It is known that anaerobic co-digestion offers a better nutrient balance for anaerobic microorganism and higher buffering capacity to prevent system acidification compared to the digestion with single feedstock (Huang et al., 2016).

3.2 Physico-chemical characteristics of anaerobic digestate

The TS and VS reduction, pH and VFA are important parameters of stability of anaerobic digestion process. Anaerobic digestion leads to the extensive degradation of organic matter in the substrate, which could be indicated by the reduction of the TS, VS content (Orzi et al., 2015). Fig. 1 and 2 shows the changes in TS and VS in slurry samples collected from biogas plants. The TS content of feedstock from Mikage was 13.8%, which was higher than 11.2% in Shikaoi. After anaerobic digestion, TS contents were significantly reduced to 7.4 and 7.1% in fermentation and sterilization tanks of Mikage biogas plant. The TS content in storage tank was further reduced to 5.1% because of the raining before sample collection. The same trends were found in VS contents in the biogas plants.

Table 1: Classifications and operating conditions of the biogas plants in this study

Parameter	Units	Mikage	Shikaoi
Digester temperature	°C	38	38
Feedstock type		Dairy manure	Dairy manure Food waste
Amount of feedstock	t/day	240	94.8
Anaerobic digesate	t/day	228	90.0
Biogas production	m^3/day	10391	3924
Power output	kW/day	750	450

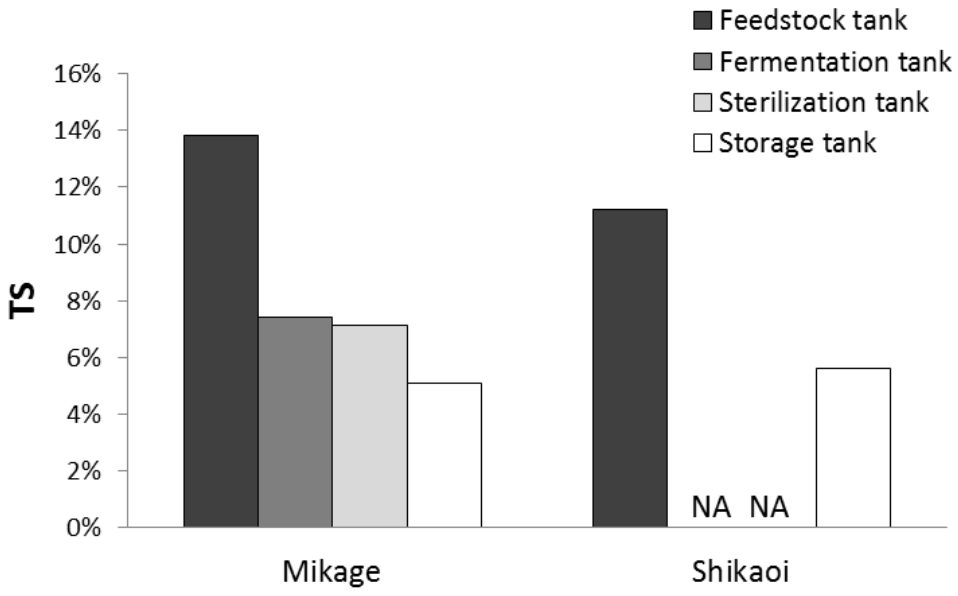

Figure 1: Change in total solids (TS) of slurry samples in two biogas plants. NA: Not analyzed

The pH data of the slurries from biogas plants are presented in Fig. 3. The pH value is one of the most important factors affecting anaerobic bacteria activity (Iwasaki et al., 2011). The pH values of feedstocks were 6.44 and 6.08 respectively, which are considered suitable for anaerobic digestion. After anaerobic digestion, the pH of digesate reached a range between 7.54 and 7.86. These results also agreed with those of other researchers (Alburquerque et al., 2012; Yamashiro et al., 2013), the pH of digestate from livestock manure tends towards the alkaline range.

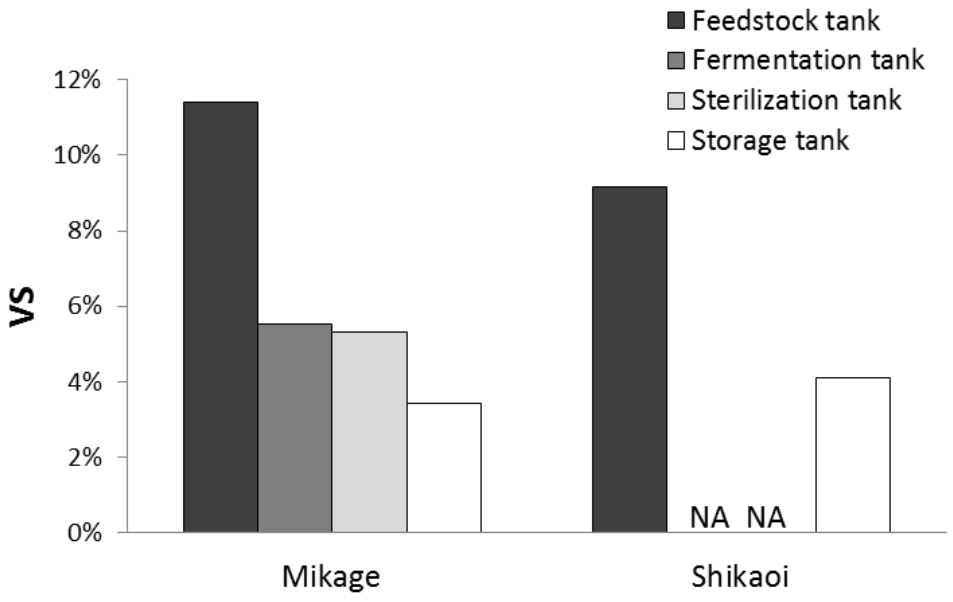

Figure 2: Change in volatile solids (VS) of slurry samples in two biogas plants. NA: Not analyzed

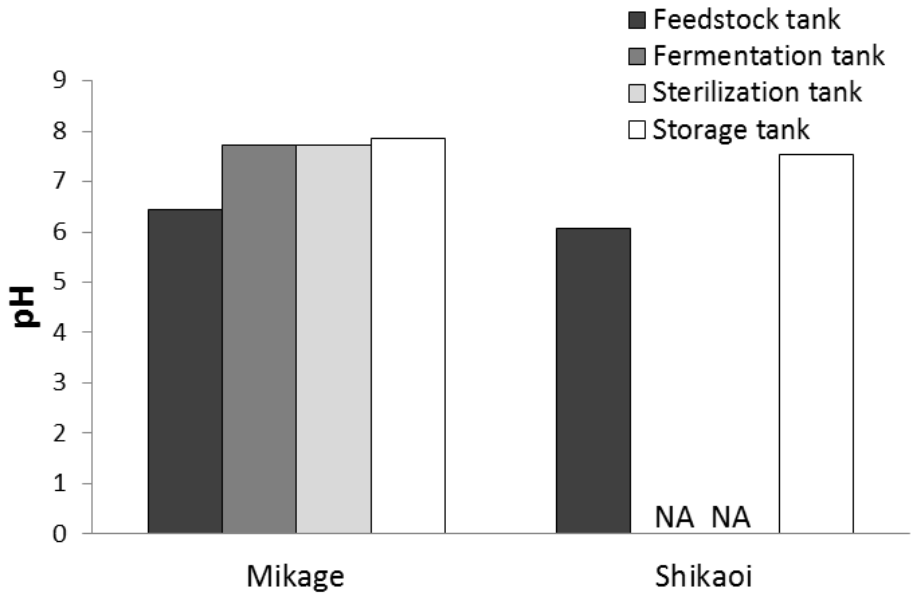

Figure 3: Change in pH values of slurry samples in two biogas plants. NA: Not analyzed

The concentrations of volatile fatty acids (VFA) in each sample are shown in Table 2. The total VFA concentrations in two feedstocks were similar: 2389.7 and 2583.0 g ml^{-1}. Acetic acid was dominant (1433.9 and 1327.6 mg L^{-1}) in feedstocks. The significant decreases in VFA were detected in two biogas plants, which indicated active consumption of VFAs by methanogens during anaerobic digestion process (Riva et al., 2016). Propionic acid concentrations (596.0 and 491.8 mg L^{-1}) were decreased to 9.7 and 11.0 mg L^{-1}or to undetectable levels. The concentrations of butyric acid in dairy manure were 359.8 and 763.7 mg $L^{-1}$and decreased to undetectable levels in each biogas plant. It is known that the VFA and pH values of the substrate affect the survival of pathogenic bacteria during anaerobic digestion (Sahlström, 2003). In this investigation, no significant difference was found in the pH values and VFA concentrations in two biogas plants. VFA also represents the largest group of odorous compounds and have been used as an odour indicator of animal manure. Leek et al. (2007) found that there was a positive linear relationship between odour emission rate (OER) of manure and the acetic acid: propionic acid ratio. In this study, the ratios of acetic acid to propionic acid were reduced by anaerobic digestion

from 3.99 to 2.68 and 2.94, which indicated that odour emission rates were decreased.

Table 2: Change in volatile fatty acid (VFA) of slurry samples in two biogas plants

		Total VFA (mg L^{-1})	Acetic acid (mg L^{-1})	Propionic acid (mg L^{-1})	Butyric acid (mg L^{-1})
Mikage	Feedstock tank	2389.7	1433.9	596.0	359.8
	Fermentation tank	41.00	30.0	11.0	ND
	Sterilization tank	39.5	29.8	9.7	ND
	Storage tank	41.8	41.8	ND	ND
Shikaoi	Feedstock tank	2583.1	1327.6	491.8	763.7
	Storage tank	27.31	27.31	ND	ND

VFA: volatile fatty acid; ND: Not detected.

3.3 Plant growth promoting bacteria (PGPB) in digestates

Substances containing PGPB are defined as bio-fertilizers which when applied to the soil, can increase plant production by supplying nutrients or promoting nutrient uptake by the plant (Vessey, 2003). However, no previous studies have focused on the PGPB in anaerobic digestate. In chapter 2, we concluded that anaerobic digestate is a large reservoir of bacteria capable of promoting plant growth. In this chapter, we investigated PGPB in anaerobic digestates from two biogas plants in Hokkaido, and the results are presented in Table 3. In Mikage biogas plant, *Bacillus* in feedstock decreased slightly from 8.21 $\log_{10}$CFU g^{-1} dry matter to a range from 7.98 to 8.00 $\log_{10}$CFU g^{-1} dry matter. Similar decrease in *Bacillus* load was also found in Shikaoi biogas plant.

Table 3: Plant growth promoting bacteria load in two biogas plants

PGPB (log_{10} CFU g^{-1} DM)		*Bacillus*	*Pseudomonas*
Mikage	Feedstock tank	8.21	6.57
	Fermentation tank	8.00	ND
	Sterilization tank	7.87	ND
	Storage tank	7.98	ND
Shikaoi	Feedstock tank	7.94	ND
	Storage tank	7.89	ND

PGPB: Plant growth promoting bacteria; CFU: Colony forming unit; DM: Dry matter; ND: Not detected.

These results are significant different from chapter 2, *Bacillus* in dairy manure increased significantly under both mesophilic and thermophilic conditions. However, the similar results about decrease in *Bacillus* load during anaerobic digestion process were found by Cao et al. (2013). Therefore, further research is recommended to ascertain the fate of *Bacillus* in the anaerobic digester. Although decreased, the *Bacillus* load in digestates were detected at a high level (7.87 to 8.12 log_{10}CFU g^{-1} dry matter), which may be attributed to spores being more robust and resistant to elevated temperatures (Kumar et al., 2012). In two biogas plants, *Pseudomonas* was detected at similar level in feedstock (6.57 to 6.66 log_{10}CFU g^{-1} dry matter), and decreased to undetectable level.

3.4 Pathogenic bacteria in digestates

In this chapter, zoonotic bacteria (*Salmonella* and *Campylobacter*) and the genera of *Enterobacteriaceae* (*Escherichia coli* and *Enterococcus*) in biogas plants were investigated, and the results are presented in Table 4.

Table 4: Pathogenic bacteria load in two biogas plants

Pathogenic bacteria (log_{10} CFU g^{-1} DM)		*E.coli*	*Enterococcus*	*Salmonella*	*Campylobacter*
Mikage	Feedstock tank	9.00	10.13	9.12	7.45
	Fermentation tank	ND	ND	ND	5.35
	Sterilization tank	ND	ND	ND	4.67
	Storage tank	ND	ND	ND	4.61
Shikaoi	Feedstock tank	7.98	9.98	10.11	7.29
	Storage tank	ND	ND	ND	6.70

CFU: Colony forming unit; DM: Dry matter; ND: Not detected.

The populations of pathogenic bacteria in two feedstocks were detected at the similar level. *E.coli*, *Enterococcus*, *Salmonella* were decreased to undetectable level after digestion in each biogas plants, which was different form the results of chapter 2. All these three bacteria were not eliminated after mesophilic and thermophilic anaerobic digestion under laboratory conditions. These results show that anaerobic digestion operated at full-scale may be more effective on inactivation of pathogenic bacteria. However, *Campylobacter* could be detected at a high level (4.61 to 6.70 log_{10} CFU g^{-1} dry matter) even in full scale biogas plants. Similarly, Kearnery et al. (1993) reported that *Campylobacter* could be detected after mesophilic anaerobic digestion, which was due to that *Campylobacter* was the most resistant bacteria during anaerobic digestion (Kearney et al., 1993). Therefore, it is important that appropriate management practices are implemented to minimize the sanitary risks of bacterial transfer to agricultural land from the application of anaerobic digestates.

4. Conclusion

Plant growth promoting bacteria and pathogenic bacteria in biogas plants were investigated in this study. All detected bacteria were reduced to undetectable level expect *Bacillus* and *Campylobacter*. The presence of *Bacillus* also makes anaerobic digestate a potential bio-fertilizer. However, *Campylobacter* residue is considered a possible source of environmental contamination. Therefore, it is important that appropriate management practices are implemented to minimize the sanitary risks of bacterial transfer to agricultural land from the application of anaerobic digestate.

General Discussion

The demand for eco-friendly energy is growing worldwide as the consumption of fossil fuel causes an increase in the concentration of greenhouse gases in the atmosphere. Improper livestock manure disposal are considered detrimental to the environment and hazardous to public health (Yamashiro et al., 2013). Generation of biogas from the anaerobic digestion of livestock manure is a bio-chemical process that provides sustainable energy and reduces the environmental risks associated with livestock manure management. Anaerobic digestion also produces a nutrient-rich residue, which is called digestate. The sustainability anaerobic digestion process depends highly on the appropriate disposal of digestate produced. Recycling digestate as an organic fertilizer is considered the most suitable utilization of digestate, as it recycles plant nutrients and reduces the consumption of mineral fertilizers (Holm-Nielsen et al., 2009). The fertilizer properties of digestate highly depend on the composition of feedstock and operating conditions of digestion (Alburquerque et al., 2012). In recent years, the fertilizer properties of digestate in various feedstocks have been widely investigated, whereas very limited information is available related to the effect of operating conditions such as temperature on fertilizer properties of anaerobic digestate. In addition, the effect of microorganisms in organic fertilizers has recently attracted attention, especially for the plant growth promoting effects of *Bacillus* and *Pseudomonas* species, which have been widely researched. However, anaerobic digestates are host to numerous plant growth promoting bacteria (PGPB) and little attention has been focused on the isolation and characterization of PGPB from anaerobic digestate. Therefore, the focus of this PhD thesis was on the fertilizer properties of anaerobic digestate from mesophilic and thermophilic digestion, and special attention was paid to isolation and characterization of PGPB from anaerobic digestate.

1. Effects of temperature on fertilizer properties of anaerobic digestate

Anaerobic digestion could be conducted under mesophilic or thermophilic condition according to different aims: mesophilic condition costs lower energy and shows a higher stability process, while thermophilic condition leads to more rapid convert of feedstock and a higher reduction of the pathogenic bacteria (Micolucci et al., 2016). In chapter 1, the

effects of mesophilic and thermophilic conditions on fertilizer properties of anaerobic digestate were investigated. The results show that the temperature has no effects on total nutrients in anaerobic digestates, however, thermophilic condition lead to a higher concentration of inorganic plant-available nutrients (NH_4^+-N), which is attributed to the higher convert rate of organic matter under thermophilic condition.

2. Plan growth promoting bacteria (PGPB) in anaerobic digestate

Substances containing PGPB are defined as bio-fertilizers which when applied to the soil, increase plant production by supplying nutrients or promoting nutrient uptake by the plant. In this PhD thesis, the fertilizer properties of anaerobic digestate were mainly evaluated according to PGPB contents and activity. In chapter 2, anaerobic digestates from different temperature of laboratory scale digestion show significant difference in PGPB contents and activity. The results showed that *Bacillus* in dairy manure increased by 5.8-fold and 1.1-fold, from 5.5 $\log_{10}$CFU/g-dry matter to 6.3 and 5.8 $\log_{10}$CFU/g-dry matter under mesophilic and thermophilic conditions, respectively. The contents of *Pseudomonas* in thermophilic digestate were also higher than that in mesophilic digestate. Moreover, 25 tested bacterial isolates from the mesophilic digestate showed plant growth promoting characteristics, which was significantly higher than 12 isolates from the thermophilic digestate. These results indicated that temperature affected PGPB content in anaerobic digestates. Mesophilic digestate may be more effective bio-fertilizer than thermophilic digestate.

3. Environmental risks related to anaerobic digestate application

Anaerobic digestates must be proven hygienically safe before they can be applied to agricultural soils. In recent years, many researches have investigated the environmental risks associated with anaerobic digestate, and main contaminants were pathogenic bacteria and heavy metals.

3.1 Pathogenic bacteria

Livestock manure is considered to contain pathogenic bacteria that might pose a

health risk for humans and animals. Pathogenic bacteria are considered to be reduced during the anaerobic digestion, but the reduction rate depends on many factors, such as the fermentation temperature, fermentation time, and initial number of bacterial species (Sahlström, 2003). In chapter 2, the reduction rates of pathogenic bacteria were above 90% after thermophilic digestion, and the maximum rate was 99.7% for *E. coli*, which was higher than after mesophilic digestion (a minimum of 73.2% for *Campylobacter* and maximum of 96.9% for *E. coli*). The higher contents of pathogenic bacteria in mesophilic digestate were detected, which was in accordance with the contents of plant growth promoting *Bacillus* and *Pseudomonas*. Although, mesophilic digestate shows higher PGPB contents and activity and may be more effective bio-fertilizer, however, the higher residue of pathogenic bacteria may be also an environmental risk.

3.2 Heavy metals

In recent years, agricultural application of anaerobic digestate has caused public concern due to its increased heavy metals content. The reason for the increasing concern is that heavy metals are used as feed additives to promote livestock growth, and their contents are known to be increasing in livestock manure, which is used as a substrate for anaerobic digestion (Zhu and Guo, 2014). In chapter 2, the changes of heavy metals content after anaerobic digestion were investigated. The concentrations of these heavy metals in dry matter were typically higher in digestates than in dairy manure, due to weight loss in anaerobic digestion process following organic matter conversion and release of biogas (Dong et al., 2013; Micolucci et al., 2016). The increase in total concentrations of heavy metals in digestates is likely to show an increased risk to the environment. However, the heavy metal concentrations of digestates in this study were lower than in other feedstocks, such as pig slurry, sewage sludge and biowaste (Fruits, vegetables and kitchen waste). These differences indicate that the level of heavy metals in digestates is highly dependent on their concentrations in the feedstock. Therefore, it is imperative to remove these heavy metals before the application of anaerobic digestate when the feedstock used, contains high contents of heavy metal.

4. Investigation of plant growth promoting bacteria and pathogenic bacteria in biogas plants, Hokkaido

In chapter 3, the fates of plant growth promoting bacteria and pathogenic bacteria in two full scale biogas plants in Hokkaido were investigated. The results showed that *Bacillus* in feedstock decreased after anaerobic digestion in full scale biogas plants and *Pseudomonas* was eliminated, which were different from the results of chapter 2. Furthermore, pathogenic bacteria except *Campylobacter* were eliminated. These results indicate that the difference in bacteria reduction rate between laboratory scale and full scale anaerobic digestion. Treatment of livestock manure in full scale biogas plants is an effective method of reducing environmental risks associated with pathogenic bacteria. However, high residue of *Campylobacter* was detected after full scale anaerobic digestion, this result was in accordance with chapter, which was due to that *Campylobacter* was the most resistant bacteria during anaerobic digestion (Kearney et al., 1993). Therefore, it is necessary to take appropriate management practices to minimize the sanitary risks of bacterial transfer to agricultural land.

General Summary

Current attentions in agriculture are focused on the reduction in use of mineral fertilizers, compelling the research for alternatives. Anaerobic digestate from biogas plants treating livestock is an ideal organic fertilizer as it recycles plant nutrients in feedstock and reduces the consumption of fossil fuel-dependent mineral fertilizers. So far, most of studies related to fertilizer properties of anaerobic digestate are focused on different feedstock, and limited information of available on the operating conditions of anaerobic digesion. Generally, anaerobic digestion is conducted under mesophilic temperature as it low cost and high process stability. However, anaerobic digestion under thermophilic temperature has attracted attentions as its high conversion rate of organic wastes and reduction rate of pathogenic bacteria. Therefore, the objectives of this PhD thesis were to investigate the effects of temperature on fertilizer properties of anaerobic digestates. Plant nutrients, pathogenic bacteria and heavy metals were detected in feedstock and digestates from mesophilic and thermophilic anaerobic digestors. Moreover, the fertilizer properties of anaerobic digestates were further evaluated according the contents and activities of plant growth promoting bacteria (PGPB). PGPB represent a wide variety of bacteria, which can occupy the rhizosphere of many plant species and have beneficial effects on plant growth directly by assisting in nutrients acquisition or providing phytohormones, or indirectly decreasing inhibitory effects of various fungal pathogens.

The results show that the temperature has no effects on total nutrients in anaerobic digestates, but has significant effects on the contents of plant growth promoting bacteria and pathogenic bacteria in digestate samples. Plant growth promoting *Bacillu*s was detected at a higher content in digestate than in feedstock, especially in mesophilic digestate. *Bacillus* species have been widely reported to have antifungal activity against many phytopathogen. Therefore, further investigation should focus on the potential of application of anaerobic digestate as bio-pesticides. Along with PGPB, mesophilic digestate also contained higher level of pathogenic bacteria than thermophilic digesate. Although, high contents of PGPB are beneficial for agricultural plants, high contents of pathogenic bacteria in mesophilic digestate should also been concerned before application.

The *Bacillus* and *Pseudomonas* isolates in chapter 2 showed various plant growth

promoting characteristics and antifungal activity. Furthermore, inoculation with these bacteria also significantly promoted growth of wheat seedlings. Therefore, these isolates could be further researched for plant growth promoting characteristics and applied as plant growth promoting inoculants.

The PGPB and pathogenic bacteria in full scale biogas plants were investigated in chapter 3. The results showed that *Bacillus* was reduced after anaerobic digestion, which was different from chapter 2, but was detected at a high content in anaerobic digesate. Therefore, digestate from full scale biogas plants also show potential as a bio-fertilizer. Most of detected pathogenic bacteria were eliminated in full scale biogas plants. In contrast, residues of detected pathogenic bacteria were detected after laboratory scale anaerobic digestion, even under thermophilic condition. This difference shows that treatment at biogas plants is an effective measure to reduce environmental risks related to pathogenic bacteria in livestock manure. However, *Campylobacter* residue was detected after both laboratory scale and full scale anaerobic digestion, which is considered a possible source of environmental contamination. Therefore, the appropriate management practices are necessary to minimize the sanitary risks of bacterial transfer to agricultural land.

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要旨

畜産業から排出される大量の家畜ふん尿は、不適切に管理または処理された場合、環境に対し潜在的な汚染源となる。有性機廃棄物をバイオガスプラントで嫌気発酵処理とは、有機廃棄物のリサイクル、再生可能エネルギーの生産、温室効果ガスの削減、バイオ肥料の提供の観点から最も適切な方法と思われる。近年、バイオガスプラントは注目を集めており、世界中で多くのバイオガスプラントが造られている。嫌気発酵処理後の消化残留物は消化液と呼ばれ、バイオガスプラントの増加により、その量も急激に増加した。バイオガスプラントの持続性を保つには、嫌気消化液の適切な利用促進が重要である。

現在、北海道では約 330 基のバイオガスプラントが稼働しており、これらからの嫌気消化液はバイオ肥料として使用されている。嫌気消化液は無機態の栄養素を多く含み、無機肥料の代わりに使用される。バイオガスプラントにおける嫌気発酵は、中温（37 °C 前後）または高温（55 °C 前後）で行うことができる。中温発酵はエネルギー投入コストが低く、発酵安定性が高いという利点を持ち、高温発酵は消化速度が速く、病原体の減少率が高い。しかし、発酵温度が嫌気消化液の肥料特性に与える影響についてはほとんど知られていない。近年、嫌気性消化液の肥料利用は、病原菌や重金属に関するリスクに関心が高まっている。さらに、有機肥料中の微生物の影響が注目され、特に *Bacillus* および *Pseudomonas* 属菌の植物生長促進効果について広く研究が行われている。このような植物成長促進細菌は、多くの植物種の根圏を占有し、直接的に栄養分、植物ホルモンを供給することによって、間接的に真菌病原体の増殖を抑制し、植物の生育を促進する。

本博士論文は、家畜ふん尿を中温および高温処理した嫌気消化液の肥料特性、特に嫌気消化液中の植物成長促進細菌について明らかにすることと、病原菌と重金属に関する環境リスクを評価することの 2 つを目的とした。

第 1 章では、実験室スケールの嫌気発酵槽からの中温および高温嫌気消化液の N および NH_4^+-N、P（P_2O_5）、K（K_2O）、Ca（CaO）および Mg（MgO）の含有量を分析した。その結果、2 つの消化液は同等の植物栄養素を含んでいるが、NH_4^+-N は高温消化液は中温消化液より高い値であった。更に、病原菌（*Salmonella*、*Campylobacter*、*Escherichia coli*、

Enterococcus）および重金属（Mn、Zn、Cu、Ni）の含有量を分析し、環境リスクを評価した。高温条件下では病原菌の減少率は 90％以上で、中温条件下より高いことが認められ、高温消化液の安全性が示された。また、乳牛ふん尿からの消化液では重金属含有量が低いことが明らかとなった。

第 2 章では、中温および高温嫌気消化液から、*Bacillus* と *Pseudomonas* を分離し、3 つの異なる植物成長促進特性：リン酸可溶化能、シデロフォアおよび植物ホルモン産生能、抗真菌活性を持つ細菌を各消化液から分離した。これらの分離株をコムギ種子に接種して植物成長促進効果を評価した。その結果、実験室スケールの嫌気発酵処理では *Bacillus* 属菌が増加しており、中温消化液から分離した 25 株、高温消化液から分離した 12 株の *Bacillus* および *Pseudomonas* は全て植物成長促進作用を示し、さらに *Bacillus* 株では抗真菌活性が認められた。

第 3 章では、北海道内の 2 つフルスケールのバイオガスプラントの嫌気消化液中の植物成長促進細菌と病原菌に焦点を当てた。各バイオガスプラントの原料槽および貯蔵槽から嫌気消化液を採取し、*Bacillus* と *Pseudomonas* の植物成長促進細菌と *Salmonella*、*Campylobacter*、*Escherichia coli*、*Enterococcus* の病原菌の含有量を測定した。その結果、フルスケールの嫌気発酵によって *Bacillus* 属菌は減少し、さらに、*Campylobacter* 属菌以外の病原菌の減滅が認められた。これらの結果は、実験室スケールとフルスケールの嫌気発酵の細菌減滅効果に差があることを示している。しかし、*Bacillus* 属菌はどちらのバイオガスプラントの消化液でも比較的多く検出され、消化液が植物成長促進効果を持つバイオ肥料として有用である可能性が示された。一方、実験室スケールとフルスケールの嫌気発酵の両方でも *Campylobacter* 属菌の残存が認められたため、危害要因となる可能性が考えられた。

本博士論文の研究成果をまとめると、（1）発酵温度が消化液の肥料特性に影響を与える主要な決定因子であり、高温嫌気発酵では、高い無機栄養素（NH_4^+-N）の含有量と病原菌の減滅効果が認められた。しかし、中温消化液は植物成長促進細菌（*Bacillus*、

Pseudomonas）の含有量と割合が高かった。（2）嫌気消化液は植物成長促進細菌を多く含み、これらの細菌は植物の根圏に定着することで植物の栄養素利用を助けるとともに植物病原菌に対する感染防除を行う。（3）乳牛ふん尿を原料とした嫌気消化液中の重金属は他の有機性廃棄物を原料とした場合よりも低かった。（4）フルスケールのバイオガスプラントでは、*Bacillus* および *Campylobacter* 以外の細菌が減滅した。*Campylobacter* の残存は消化液利用の危害要因となる可能性が考えられたため、より効果的な処理方法が今後の課題である。