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Anaerobic co-digestion of dairy manure, meat and bone meal, and crude glycerol under mesophilic conditions: Synergistic effect and kinetic studies

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ABSTRACT

Anaerobic digestion is a potential renewable energy, climate independent and robust technology, which is able to treat different kinds of organic wastes and by-products. This study investigated the anaerobic co-digestion of meat and bone meal (MBM) with dairy manure (DM) and crude glycerol (CG). Three sets of batch experiments were conducted at mesophilic condition; one set of anaerobic mono-digestion and two sets of anaerobic co-digestion. In experiment I, each substrate was mono-digested at inoculum to substrate ratio of 1. In experiment II, MBM and DM were co-digested at ratios of 1.0:1.0, 1.0:2.0, 1.0:1.0, and 2.0:1.0, while in experiment III CG was co-digested with MBM at ratios of 1.0:3.0, 1.0:1.0 and 3.0:1.0, at a fixed amount of DM. The results of anaerobic mono-digestion showed that CG produced the highest methane yield (0.48 L/gVS) followed by MBM (0.41 L/gVS) and DM (0.17 L/gVS). In the anaerobic co-digestions, methane yield increased with the increase of MBM content, while it increased together with CG content. The kinetic studies showed that the physico-chemical characteristics of the co-digested substrates influenced hydrolysis rate constant and lag-phase, which increased with the increase of CG content. However, synergistic effect was decreased when MBM content was increased, whereas the opposite was observed to that with CG. Therefore, carbon to nitrogen ratio was an important parameter determining synergistic effect in anaerobic co-digestion, while the physico-chemical characteristics influenced the hydrolysis rate constant and lag-phase.

Introduction

Since the 1900s meat and bone meal (MBM) has been utilized in livestock industries as a source of protein (Swisher, 2006). At the beginning of the 1980s, the demand of rendered animal proteins to animal fed has increased dramatically until the 1990s, when bovine spongiform encephalopathy (BSE) outbreak in many countries, which is associated with MBM contaminated with a protein called "prions" (Swisher, 2006; Conesa et al., 2005). As a consequence, the utilization of MBM in animal feed was restricted in the European Union in 2000 (Mondini et al., 2008), while it was in 2001 for Japan after the first case of BSE outbreak (Sugiura et al., 2014). Thereafter, MBM was considered as a waste and required disposal technologies to cope with the million tons of MBM produced annually (e.g., 3 million tons in Europe (Cyr and Ludmann, 2006) and 4 million tons in the U.S. (Swisher, 2006)). Incineration or melting in a cupola furnace is among the most common disposal technology for MBM, which was widely used (Conesa et al., 2005). However, due to the large amount of MBM produced from rendering plant, the insufficiency of incinerator capacity was a challenge. Therefore, other alternatives were considered such as the use of MBM in cement manufacturers and in agriculture (Mondini et al., 2008; Cyr and Ludmann, 2006). In May 2013 Japan was declared as a BSE free country (OIE, 2013), and hence Japanese Government allowed the use of MBM as organic fertilizer since June 2014.

Since MBM has a calorific value of 17.1 MJ/kg (dry weight) (Soni et al., 2009), its utilization in anaerobic digestion is of interest to recover energy in the form of methane. The utilization of MBM in anaerobic digestion has been reported by Wu et al. at different total solid contents (Wu et al., 2009a) and thermochemical pretreatments (Wu et al., 2009b). They found that the highest methane yields were observed at a MBM solid contents of 5% (0.38–0.45 L/gVS$_{removed}$) (Wu et al., 2009a), and with alkaline pretreatment (NaOH) at 131 °C (0.46–0.56 L/gVS MBM) (Wu et al., 2009b). However, anaerobic mono-digestion of MBM is not always practicable and is a challenging process due to the accumulation of free ammonia nitrogen (Wu et al., 2009a), which can penetrate into microbial cells and disturb cellular homeostasis (Kayhanian, 1999). Different approaches have been used to overcome ammonia inhibition in anaerobic digestion such as dilution of substrates (Kayhanian, 1999; Hejnfelt and Angelidaki, 2009), the combination of digester with an electrochemical

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system to extract ammonium (Desloover et al., 2015), the combination of AD process with a microbial electrolysis cell coupled to ammonia stripping and adsorption unit to recirculate effluent (Cerrillo et al., 2016), and optimization of feed composition and carbon to nitrogen (C/N) ratio (Nurliyana et al., 2015; Wang et al., 2012). Among them, the adjustment of C/N ratio through anaerobic co-digestion sounds to be the most environmentally friendly and economically profitable to address ammonia inhibition because it does not reduce the digester performance (in the case of dilution) or required additional investment for another unit (in the case of microbial electrolysis cell or electrochemical system). To the best of our knowledge, anaerobic co-digestion of MBM with low or high C/N ratio substrates has not been yet investigated.

Aside C/N ratio adjustment, co-digestion of different substrates helps to stabilize anaerobic digestion process by supplying optimal moisture content and pH, enhancing buffer capacity, balancing essential nutrients and trace metals, and diluting potential inhibitory or toxic compounds (Esposito et al., 2012). Dairy manure (DM) is a high moisture substrate (more than 87%), and is recognized to be an excellent "carrier" substrate that has been used in different anaerobic co-digestion processes as the base substrate (Andriamanohiarisoamanana et al., 2016; Atandi and Rahman, 2012; Angelidaki and Ellegaard, 2003), as it is widely available (Yabe, 2013) and contains the biomass population to produce methane. It can be, therefore, co-digested with MBM, which is a low moisture substrate (less than 3%). However, the co-digestion of low C/N ratio substrates (DM and MBM) may be challenged by the exceedance of nitrogen for microorganism's growth and, probably, leads to the accumulation of free ammonia nitrogen.

Crude glycerol (CG), which is a by-product of biodiesel making companies, is commonly used as a co-substrate in anaerobic co-digestion. It has a C/N ratio ranging between 248:1 and 275:1 (Castrillón et al., 2013; Chen et al., 2008), and has been used in anaerobic co-digestion with nitrogen-rich substrate to adjust to an optimum C/N ratio of 16–33 (Mata-Alvarez et al., 2014) and to boost biogas production (Wohlgemut et al., 2011; Fountoulakis et al., 2010). Various nitrogen-rich organic wastes have been anaerobically co-digested with CG such as cattle manure (Castrillón et al., 2013), swine manure (Wohlgemut et al., 2011) and sewage (Fountoulakis et al., 2010). A significant improvement of biogas production (four times) was observed by Kato et al. (2010) at an addition of CG at ratio of 6% in a laboratory-scale experiments of anaerobic co-digestion with DM. Similarly, an improvement by about two times was observed by Andriamanohiarisoamanana et al. (2016) when DM was co-digested with CG in a thermophilic farm-scale biogas plant. Because MBM has low C/N ratio, the anaerobic co-digestion of MBM with CG is, therefore, an alternative to adjust C/N ratio to an optimum value, to increase methane production from MBM and also to properly manage the projected 4.6 million tons of CG in 2020 (Viana et al., 2012).

The main objective of this study was to investigate the effects of MBM on the improvement of methane yield and process stability in anaerobic co-digestion of DM and CG under mesophilic conditions. The first specific objective was to determine the methane yield of the anaerobic mono-digestion of DM, MBM and CG (experiment I). The second specific objective was to investigate the methane yield and process stability of anaerobic co-digestion of DM and MBM (experiment II). The third specific objective was to determine the effects of anaerobic co-digestion of MBM and CG using DM as main substrate on process performances (experiment III). To obtain useful information for academic and practical applications, experimental and mathematical approaches were undertaken. Particularly, focus was given on synergistic effect and kinetic studies.

Materials and method

Materials

Fresh dairy manure (DM) was obtained early in the morning from the free stall barn of 70 lactating Holstein cows located in Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan. DM was stored at 4 °C before its use on the next day. Meat and bone meal (MBM) was obtained from a rendering company in Hokkaido. MBM was kept in a freezer at −20 °C until use in order to prevent any change in terms of characteristics and components. The characteristics and components of MBM are shown in Table 1. Crude glycerol (CG) was obtained from a local biodiesel-making company that transforms used cooking oils into biodiesel fuel for local transportation, especially busses and taxis. Methanol was used for transesterification with potassium hydroxide as a catalyst. The characteristics of DM and CG are reported elsewhere (Andriamanohiarisoamanana et al., 2016).

Inoculum was obtained from an active mesophilic biogas reactor treating primarily dairy manure. Inoculum was collected a day before the start of experiment and kept at 4 °C until use. All substrates and inoculum were characterized by measuring total solids content (TS) and volatile solids content (VS) before the start of the experiments.

Experimental setup

Laboratory scale batch experiments were conducted using 1 L laboratory-scale batch digesters, made from polypropylene, with working volume of 600 mL. Based on the objectives of this study, three groups of batch experiments were conducted, namely anaerobic mono-digestion of DM, MBM and CG (experiment I), anaerobic co-digestion of DM and MBM (experiment II) and anaerobic co-digestion of DM, MBM and CG (experiment III). Inoculum was pre-incubated at 38 °C for 3 days prior to the start of experiments to acclimatize inoculum to the new environment and to complete the digestion of remaining organic substrate (degassing) before the addition of new substrate.

Before the start of anaerobic co-digestion, anaerobic mono-digestion of each substrate (DM, MBM and CG) was conducted in experiment I. Samples were prepared and fed into digesters (D1–D3) to obtain an inoculum to substrate ratio of 1.0:1.0 (gVS inoculum:gVS substrate). Deionized water was added to bring the final volume to 600 mL. In experiment II, anaerobic co-digestion of DM and MBM were investigated by varying the amount of MBM from 1 to 20 g, while the amount of DM was fixed at 100 g. Before weighting the samples, the VS of DM was adjusted to 8%. 100 g of DM was mixed with 1, 5, 10, and 20 g of MBM to obtain a MBM to DM ratio of 1.0:10.0, 1.0:2.0, 1.0:1.0, and 2.0:1.0 (gVS MBM:gVS DM), respectively, and fed into digesters (D4–D7). The inoculum to substrate (DM + MBM) ratio was fixed at 1.0:1.0, and deionized water was added to bring the target volume of 600 mL. In experiment III, the main focus was on anaerobic co-digestion of MBM and CG, while DM was utilized as the base substrate (Atandi and Rahman, 2012). The amount of CG and MBM was varied to three levels to obtain a CG and MBM ratio of 1.0:3.0, 1.0:1.0 and 3.0:1.0 (gVS CG:gVS MBM), while the amount of DM was fixed at 100 g, which represents a DM and substrate (CG + MBM) ratio of 1.0:1.0. The inoculum to substrate (DM + MBM + CG) ratio was fixed at 1.0:1.0, and deionized water was added to bring the target volume of 600 mL and fed into digesters (D8–D10). Blank test digester (D0) containing only inoculum was conducted in order to adjust the biogas

Table 1
Characteristics of meat and bone meal.

Parameters	Units	Meat and bone meal
Total solids content	% (w/w)	98.46
Moisture content	% (w/w)	1.54
Volatile solids	% (w/w)	68.47
Higher heating value	MJ/kg	17.20
Lower heating value	MJ/kg	16.00
n-Hexane extracts	% (w/w)	11.15
Phosphorus	% (w/w)	4.07
Potassium	% (w/w)	0.48
Nitrogen	% (w/w)	10.52
Carbon	% (w/w)	44.09
Hydrogen	% (w/w)	5.22
C/N ratio		4.19

obtained in the mixture inoculum-substrate. Details of experimental design are illustrated in Table 2. All digesters were sealed and incubated in water baths at a controlled temperature of 38 °C. Digesters are manually shaken every 24 h throughout the experimental period, and the quantity of biogas was measured daily (Andriamanohiarisoamanana et al., 2017) along with the gas compositions. The incubation period was finished until biogas emitted from the digester did not show significant fluctuation, which was about 30 days. Aliquot of samples from each digester were taken before and after the digestion process and were analyzed for total solids (TS), volatile solids (VS) and volatile fatty acids (VFA). pH was also measured before and after digestion process.

Analytical procedures

TS and VS were measured according to the standard methods (part 2540G) (APHA, 2005). Gas compositions were determined using a GC-14A (Shimadzu, Japan) equipped with a thermal conductivity detector (stainless column and Porapak Q packing). The operational temperatures of the injector port, the column and the detector were 220, 150 and 220 °C, respectively. Argon was the carrier gas at a flow rate of 50 mL/min. Individual VFA (formic acid, acetic acid, propionic acid, and butyric acid) of samples were analyzed with a high-performance liquid chromatograph (HPLC, Shimadzu LC-10 AD, Japan) with a Shim-Pack SCR-102H column. The details of the procedure can be found elsewhere (Kimura et al., 1994).

Data analysis and kinetic studies

To compare the results obtained in this study with the results reported in literatures, the gas volume was adjusted at standard temperature and pressure (STP) conditions (Andriamanohiarisoamanana et al., 2017).

The C/N ratio of mixture substrate was calculated based on the individual carbon and nitrogen percentage of individual substrate as shown in Eq. (1).

$$\beta = \sum_{i=1}^{n} \frac{V_i C_i T_i}{V_i N_i T_i} \quad (1)$$

where β is the C/N ratio of the mixture substrate; n is the number of co-digested substrates; i is the individual substrate; V_i is the mass of substrate added (g); C_i is the percentage of carbon in the substrate (%), N_i is the percentage of nitrogen in the substrate (%); T_i is the total solids content (%).

First-order kinetic model (Eq. (2)) was used to determine the hydrolysis rate constant (Angelidaki et al., 2009), while modified Gompertz model (Eq. (3)) was used to calculate the lag-phase and methane production potential (Zhu et al., 2014).

$$C_t = C_{max} \cdot (1 - exp(-kt)) \quad (2)$$

where, t (day) is the time, C_{max} (L/kgVS$_{added}$) is the cumulative methane yield obtained in 30 days, C_t (L/kgVS$_{added}$/day) is the methane yield obtained at time t, and k is the hydrolysis rate constant.

$$M_{(t)} = M_0 \cdot \exp\left\{ - exp\left[\frac{R_{max} \cdot e}{M_0} (\lambda - t) + 1 \right] \right\} \quad (3)$$

where, $M_{(t)}$ (L/kg VS$_{added}$) is the cumulative methane yield at time t, e is exp.(1) = 2.71828, R_{max} (L/kg VS$_{added}$/day) is the maximum specific methane production rate, M_0 (L/kg VS$_{added}$) is the methane production potential, and λ (day) is the lag phase time. The parameters in this equation (M_0, R_{max} and λ) were estimated by the least squares method using the Solver Function of Microsoft® Office Excel 2010 (Ohuchi et al., 2014).

Time period to produce 90% of methane yield (T_{90}) and the time period for effective methane production (T_{ef}) can be calculated according to Eq. (4) and Eq. (5), respectively (Kafle and Kim, 2013).

$$T_{90} = \lambda + 3.25 \cdot \frac{M_0}{R_{max} \cdot e} \quad (4)$$

$$T_{ef} = T_{90} - \lambda \quad (5)$$

Synergistic or antagonistic effects of the co-digestion of substrate mixtures are expressed by the weighted specific methane yield (*WSMY*), which is the sum of the individual contributions of each substrate during the mono-digestion (experiment I) (Eq. (6)) (Labatut et al., 2011).

$$WSMY = \sum_{i=1}^{n} \frac{M_i S_i}{S_0} \quad (6)$$

where n is the number of co-digested substrates; M_i is the individual methane yield of substrate (L/gVS); S_i is the added VS of individual substrate in the mixture (g); S_0 is the total VS of mixture substrate (g).

Statistical analysis

Each experimental treatment was conducted in triplicate, and the results are presented as means of each parameter. Significant difference was determined by analysis of variance, which was preformed using the GLM procedure of SAS (SAS Inst., Inc., Cary, NC). Significant difference was determined at p-value of less than 5%.

Results and discussions

Anaerobic mono-digestion of DM, MBM and CG (experiment I)

The cumulative biogas yield and cumulative methane yield during the anaerobic mono-digestion of DM, MBM and CG are shown in Fig. 1. From all digesters, the biogas production started from the first day of

Table 2
Experimental design.

	Digester	DM (%VS$_{added}$)	MBM (%VS$_{added}$)	CG (%VS$_{added}$)	Ratio[a]	OLR (gVS/L)	Total TS$_{digester}$ (%)	Total VS$_{digester}$ (%)
Experiment I	D1	100	0	0	–	15	6.49	4.77
	D2	0	100	0	–	15	5.71	4.02
	D3	0	0	100	–	15	6.34	4.64
Experiment II	D4	90	10	0	1.0:10.0[b]	15	6.74	4.88
	D5	67	33	0	1.0:2.0[b]	18	7.02	5.10
	D6	50	50	0	1.0:1.0[b]	24	7.65	5.60
	D7	34	66	0	2.0:1.0[b]	36	8.93	6.75
Experiment III	D8	50	37	13	1.0:3.0[c]	27	8.99	6.77
	D9	50	25	25	1.0:1.0[c]	27	9.32	7.07
	D10	50	13	37	3.0:1.0[c]	27	9.58	7.00

[a] Ratio between substrates added based on gVS added.
[b] Ratio between dairy manure and meat and bone meal (gVS MBM: gVS DM).
[c] Ratio between meat and bone meal and crude glycerol (gVS CG: gVS MBM).

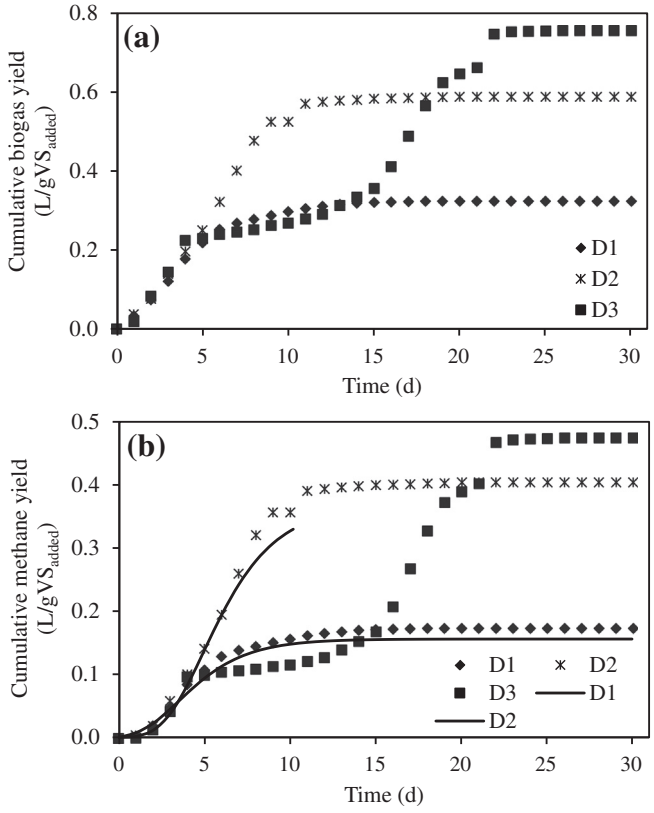

Fig. 1. Experiment I: cumulative biogas yield (a) and cumulative methane yield (b) of anaerobic mono-digestion of dairy manure (D1), meat and bone meal (D2) and crude glycerol (D3) along with the modified Gompertz plots (lines).

anaerobic digestion (Fig. 1a), which indicates a fast response of anaerobic microbial population towards the substrates. During the first 5 days, the cumulative biogas yields of the three substrates were almost similar. However, from day 6, biogas yield from D1 (DM) slowed down and achieved the plateau of biogas production from day 15. Similarly, continuous increase of biogas production was observed with D2 (MBM) until day 11, where biogas yield decreased and achieved its steady state. On the other hand, biogas yield from D3 (CG) increased continually for the first 5 days, and then slowed down until day 15, where an exponential biogas production was observed. With an average methane concentration of 54%, 64% and 59% for D1, D2 and D3 (Table 3), respectively, the patterns of methane yields (Fig. 1b) were similar to that of biogas yields. The highest cumulative methane yield was 0.48 L/gVS in D3, which is comparable to that reported in literature (Viana et al., 2012), followed by 0.41 L/gVS in D2. For D1, the cumulative methane yield was 0.17 L/gVS, which was 41% and 35% to that of D2 and D3, respectively. The methane yield of DM was in the range of specific methane yield of dairy manure reported by Labatut et al. (2011). However, it was lower than that reported by Angelidaki and Ellegaard (2003) (0.20 L/gVS), but higher than that of Møller et al. (2004) (0.15 L/gVS).

The higher specific methane yield in D2 and D3 over D1 was the result of higher methane concentrations (Table 3) and the composition of MBM and CG compared to DM. Compared to MBM and CG, which the main components are protein and lipid, DM has low methane yield because of the recalcitrant characteristic of lignocellulosic materials (fibers), which are highly present in DM (Angelidaki and Ellegaard, 2003). The methane yield of MBM was lower than that at thermophilic anaerobic digestion (Wu et al., 2009b) but comparable to that at mesophilic anaerobic digestion as shown by Wu et al. (2009a) (0.48 L/gVS$_{removed}$). The higher methane yield of CG over MBM can be explained by the higher theoretical methane yield of lipid than that of protein. In fact, gCOD (chemical oxygen demand) to gVS$_{substrate}$ ratios of protein and lipid are 1.42 and 2.90, respectively. Because 1 gCOD could produce 0.35 L of methane, the theoretical methane yield of protein and lipid is therefore, 0.50 L/gVS and 1.01 L/gVS, respectively. However, despite the fact that the theoretical methane yield of lipid (which is the dominant component in CG) is almost double than that of protein (which is the dominant component in MBM), the experimental methane yield from CG was only 17% higher than that of MBM. This may be the result of MBM processing. In this experiment, MBM was not defatted, which may contribute to the comparable methane yield of MBM with that of CG.

Although the methane yield obtained from dairy manure is lower than that of other substrates, utilizing DM as a base substrate (Atandi and Rahman, 2012) in an anaerobic co-digestion process is of interest as it has high moisture content, which is perfectly fit to be co-digested with high TS materials such as MBM and CG, has high buffer capacity and is widely available (Angelidaki and Ellegaard, 2003).

Table 3
Methane concentration and volumetric production of methane and biogas.

	Digester	Methane conc. (%)	Volumetric methane production (L/L$_{dig.ester}$)	Volumetric biogas production (L/L$_{digester}$)
Experiment I	D1	54.06	2.61	5.38
	D2	64.77	5.50	8.84
	D3	59.40	6.46	11.36
Experiment II	D4	62.91	3.89	7.96
	D5	68.61	5.37	9.13
	D6	68.52	7.54	11.96
	D7	71.91	11.63	18.26
Experiment III	D8	66.39	10.05	16.59
	D9	66.33	11.04	17.89
	D10	58.67	12.44	20.80

Anaerobic co-digestion of DM and MBM (experiment II)

Fig. 2 illustrates the cumulative biogas and methane yields during the anaerobic co-digestion of DM and MBM. At a fix OLR of DM of 13.5 gVS/L, the OLR of MBM was varied from 1.5 to 22.5 gVS/L, which represents 10–66% of the total gVS added (Table 2). Shown in Fig. 2a the highest biogas yield was observed at D4 (0.51 L/gVS), while no significant differences were observed between D5, D6 and D7 ($p > 0.05$). In contrast, the volumetric biogas production did not proportionately increase with the quantity of MBM added. In fact, the volumetric biogas production increased only by 2.3-times (from 7.96 to 18.26 L/L$_{digester}$) when the added MBM was increased by 15.0-times (from 1.5 to 22.5 gVS/L) (Table 3), indicating a slight inhibition of biogas production or low conversion of MBM into biogas at high concentration of MBM.

As illustrated in Table 3, methane concentration increased in line with MBM quantity, from 62.91% in D4 to 71.91% in D7, which obviously affected the volume of methane yield. Similar to methane concentration, in Fig. 2b, cumulative methane yield was increased with the increase of MBM concentration in the mixture substrate, and the highest methane yield was 0.30 L/gVS at D7. Compared to D4, the cumulative methane yield increased only by 4.61%, 10.90% and 17.73%, while MBM concentration increased by 2.0-, 7.0- and 15.0-times in D5, D6 and D7, respectively. This shows that the conversion rate of MBM to methane was reduced at higher MBM concentration. This may be attributed to low C/N ratio that might lead to free ammonia inhibition. In fact, the C/N ratio was decreased from 13 in D4 to 6 and 5 in D6 and D7, respectively. Nevertheless, the volumetric methane production increased between 1.4- and 3.0-times when MBM was between 2.0- and 15.0-times (Table 3), which can be attributed to the increase of OLR fed into the digesters along with MBM concentration. Moreover, as shown in Fig. 1b, the methane yield from MBM was 2.4 times higher than that of DM, which explains the increase of methane yield with MBM. However, the kinetic studies showed that lag phase of methane production increased with MBM (Table 4).

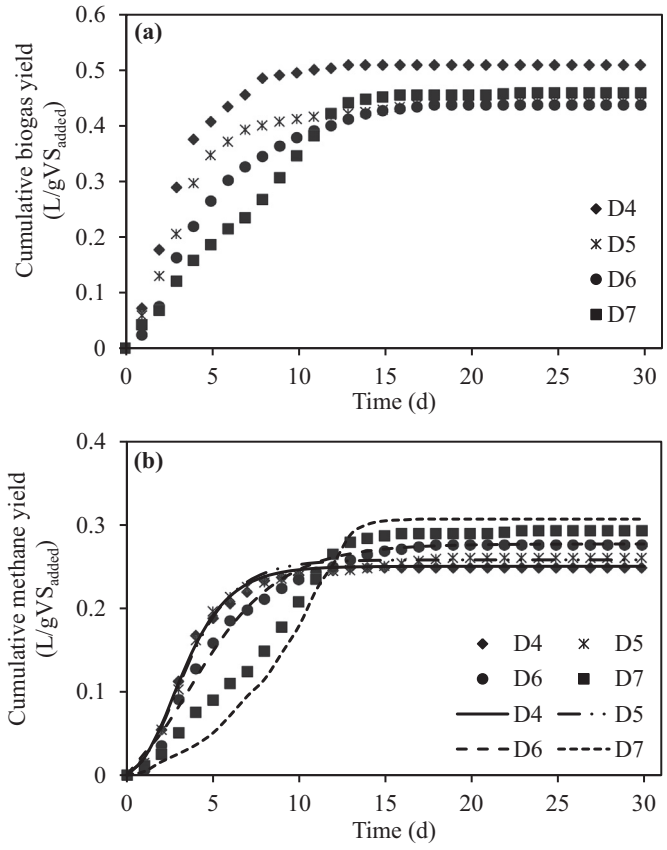

Fig. 2. Experiment II: cumulative biogas yield (a) and cumulative methane yield (b) during anaerobic co-digestion of dairy manure and meat and bone meal along with the modified Gompertz plots (lines).

The mixture of MBM, which is a protein-rich substrate (up to 45% proteins) (Lee et al., 2015; Robaina et al., 1997), with DM, which has a C/N ratio of 18:1, has led to a lower C/N ratio which may disturb or inhibit the anaerobic digestion process by the accumulation of free ammonia and volatile fatty acids (Wu et al., 2009a). Therefore, the increase of C/N ratio by mixing with carbon-rich substrate such as CG is expected not only to increase methane yield, but also to improve digestion process. However, due to the readily available characteristics of CG to acidogenic bacteria and the high impurities present in CG, such as long chain fatty acids and inorganic salts (Cl^- or SO_4^{2-}) (Viana et al., 2012), anaerobic co-digestion with CG may encounter inhibitions.

Anaerobic co-digestion of DM, MBM and CG (experiment III)

The cumulative biogas and methane yields during the anaerobic co-digestion of MBM and CG at a fix OLR of DM (13.5 gVS/L) are given in Fig. 3. As the total VS added in the digester was fixed at 27 gVS/L (Table 2), the change of cumulative biogas yields shown in Fig. 3a is only caused by the variation of CG and MBM ratios of 1.0:3.0, 1.0:1.0 and 3.0:1.0 (gVS CG: gVS MBM) for D8, D9 and D10, respectively. The highest biogas yield was 0.67 L/gVS at D10, and it decreased with increasing MBM concentration. Methane concentrations of D8 and D9 were almost similar (66%), while the lower average methane concentration was observed with D10 (Table 3). This was attributed to the slower startup of D10, where carbon dioxide was of less than 55% of the biogas for the first 8 days. However, during the stability of biogas production, the methane concentrations for the three treatments were between 67% and 75%. The improvement of methane concentration during anaerobic co-digestion is probably caused by the efficient conversion of carbon dioxide and hydrogen to methane by hydrogenotrophic methanogenic archaea, which assure the 30% of total methane production, and also by the higher biodegradability of the co-digested substrates.

Fig. 3b shows the cumulative methane yield during anaerobic co-digestion of DM, MBM and CG. In general, methane yield was increased along with CG concentration. The highest methane yield was at D10 (0.44 L/gVS), while the lowest was at D8 (0.36 L/gVS). The increase of methane yield with CG was not only caused by the improvement of C/N ratio (Silvestre et al., 2015), which was from 11 in D8 to 20 and 37 in D9 and D10, respectively, but also the specific characteristics of CG, which is highly biodegradable and has high methane production potential (0.43 L/g of glycerol (Viana et al., 2012)). This result confirms the results reported in many literatures about the role of CG to boost methane yields (Andriamanohiarisoamanana et al., 2016; Fountoulakis et al., 2010; Viana et al., 2012). In fact, compared with the results illustrated in Fig. 2b – D6, the replacement of 25, 50 and 75% (based on gVS added) of MBM to CG increased the methane yield by 29%, 39% and 57%, respectively. However, the startup of methane production was getting slower at higher CG concentration, which can be attributed to the impurities of CG and the overproduction of intermediate compounds at early stage of the digestion process as the result of the readily available CG for acidogenic bacteria. These affected negatively the activities of acitogenic bacteria and methanogenic archaea. In fact, the cellular coefficient yield of acidogens (0.15–0.17 gVS/gCOD) is almost three times that of acetogens (0.025–0.051 gVS/gCOD) or methanogens (0.020–0.054 gVS/gCOD), which led to the accumulation of intermediate compounds during digestion process (Viana et al., 2012).

Kinetic studies

The first-order kinetic model was introduced to determine the hydrolysis rate constant (k), while lag-phase (λ), maximum specific methane production rate (R_{max}) and methane production potential (M_0) were determined by modified Gompertz model. The parameters of kinetic studies are given in Table 4. The study with first-order kinetic model showed that correlation coefficients (r^2) were more than 0.90 except with D3 where r^2 was 0.62. This indicates that logarithmic methane

Table 4
Results of kinetic studies.

		First-order kinetic model		Modified Gompertz model					
	Digester	r^2	k (d^{-1})	λ(d)	r^2	R_{max} (L/gVS$_{added}$)	M_0 (L/gVS$_{added}$)	T_{90} (d)	T_{ef} (d)
Experiment I	D1	0.980	0.34	1.23	0.995	0.03	0.16	8.51	7.28
	D2	0.974	0.37	2.56	0.996	0.06	0.37	10.24	7.68
	D3	0.622	0.20	4.94	0.946	0.02	0.61	39.19	34.26
Experiment II	D4	0.995	0.39	0.85	0.996	0.05	0.25	6.67	5.82
	D5	0.945	0.26	0.87	0.990	0.05	0.26	7.03	6.16
	D6	0.967	0.27	0.60	0.990	0.03	0.28	10.31	9.71
	D7	0.934	0.29	4.32	0.954	0.04	0.33	15.08	10.76
Experiment III	D8	0.946	0.23	4.32	0.989	0.04	0.33	15.08	10.76
	D9	0.976	0.31	5.00	0.986	0.03	0.37	19.04	14.04
	D10	0.903	0.39	8.74	0.974	0.03	0.47	28.82	20.09

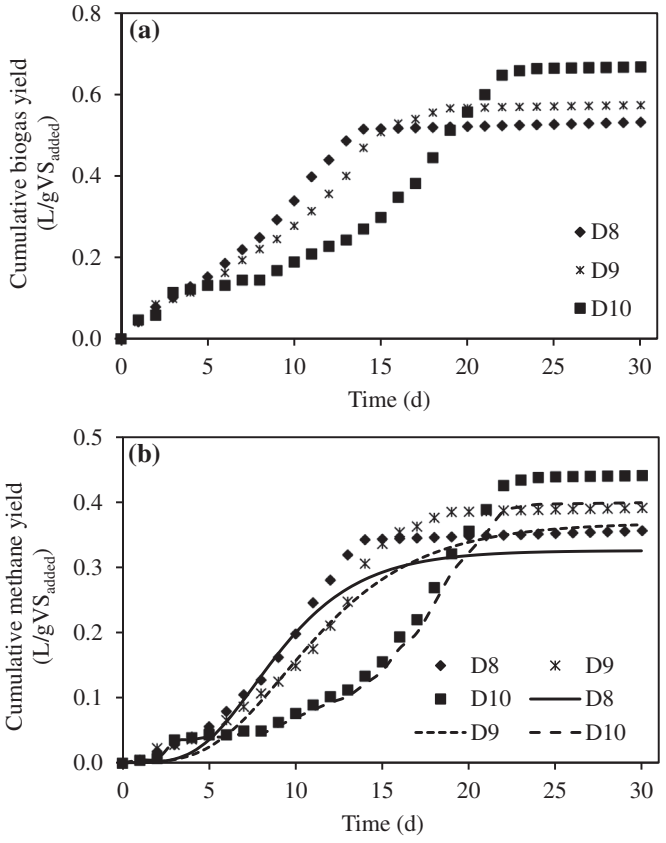

Fig. 3. Experiment III: cumulative biogas yield (a) and cumulative methane yield (b) during anaerobic co-digestion of dairy manure, meat and bone meal and crude glycerol along with the modified Gompertz plots (lines).

production of digesters followed the first-order kinetic model, except with D3 which showed a biphase profile of methane production (Andriamanohiarisoamanana et al., 2017). In experiment I, the highest *k*-value was observed with D2, while the lowest was with D3. However, in experiment III, *k* was increased with the increase of CG concentration, indicating that anaerobic co-digestion of CG increased the bioavailability of organic content in MBM and CG mixture (Zhang et al., 2014).

Lag-phase (λ) is an important parameter to determine the substrate biodegradability and utilization rate (Xie et al., 2011). In experiment I, λ of D2 and D3 were almost 2.0- and 4.0-times to that of DM, respectively. The shortest λ was in D1 (1.23 d), while the longest was in D3 (4.94 d) (Table 4). In experiment II, there was no significant difference between λ, except at D7 where λ was 4.32 d. The short λ indicates that the produced intermediates (VFAs) were rapidly converted into biogas (Astals et al., 2014), while the long λ in D7 can be attributed to the accumulation of ammonia and VFA, which is consistent with the work of Wu et al. (2009a). Therefore, for a "healthy" anaerobic co-digestion of MBM with DM, the recommended maximum MBM to DM ratio should be lower than 1.0:1.0 (gVS MBM:gVS DM), despite the fact that the C/N ratio of the mixture was far lower than the optimal (20–30 (Wang et al., 2012)). However, in a continuous fed experiment, care must be taken as daily fed of protein-rich substrate may lead to free ammonia accumulation. In experiment III, λ was increased with CG concentration, while 75% increase was observed at D10 compared to D9. The increase of λ with CG can be attributed to the ease availability of CG for acidogenic bacteria that produced substantial amount of VFA and caused severe inhibition of methanogens activity. Although the C/N ratio was improved with CG addition, the longer λ was rather caused by VFA accumulation, particularly propionic acid, than the impurities in CG (Jensen et al., 2014). Thus, in anaerobic co-digestion, C/N ratio has an important role to balance the growth of microbial

Table 5
Synergistic study of different substrates in anaerobic co-digestion.

	Digester	SMY_o (L/gVS)	*WSMY* (L/gVS)
Experiment II	D4	0.25	0.18
	D5	0.26	0.22
	D6	0.28	0.26
	D7	0.30	0.29
Experiment III	D8	0.32	0.26
	D9	0.35	0.27
	D10	0.40	0.28

SMY_0 = Specific methane yield.
WSMY = Weighted specific methane yield.

population and to produce optimum methane yield. However, the physico-chemical characteristics of the co-digested substrates are essential to assure the success of the co-digestion process.

Apart from λ and *k*, the time period for effective methane production (T_{ef}) is an important parameter, particularly for the application of the results of this study in a semi-continuous fed reactor. Although the methane production potential (M_0) increased with MBM concentration in anaerobic co-digestion with DM, the increase of MBM concentration increased T_{ef} (Table 4), indicating that longer period of time was required to produce 90% of methane and process disturbance was observed. Similar pattern was observed with experiment III. Compared to D8, the increase of CG to MBM ratio increased the T_{ef} of about 1.3- and 1.9-times at D9 and D10, respectively. Practically, the results of this study suggested that to ensure a stable anaerobic co-digestion with a higher MBM and/or CG concentration, at the same OLR, a larger reactor is required. Therefore, despite the higher cumulative methane yield obtained at higher MBM (experiment II) or CG (experiment III) concentration, T_{ef} is worth to be considered because, practically, larger reactor volume is related to higher investment and running costs of a biogas plant.

Synergistic effect study

The efficiency of anaerobic co-digestion in terms of methane yield can be expressed by the synergistic or antagonistic effect study. The synergistic or antagonistic effects of the anaerobic co-digestion was calculated, and presented in Table 5 and illustrated in Fig. 4. Weighted specific methane yield (*WSMY*) was calculated based on the results obtained in experiment I (Eq. (6)). The synergistic or antagonistic effects of the co-digestion substrates can be determined by the difference between specific methane yield (SMY_0, methane yield obtained in experiment II and III) and *WSMY* (Fig. 4). There is a synergistic effect

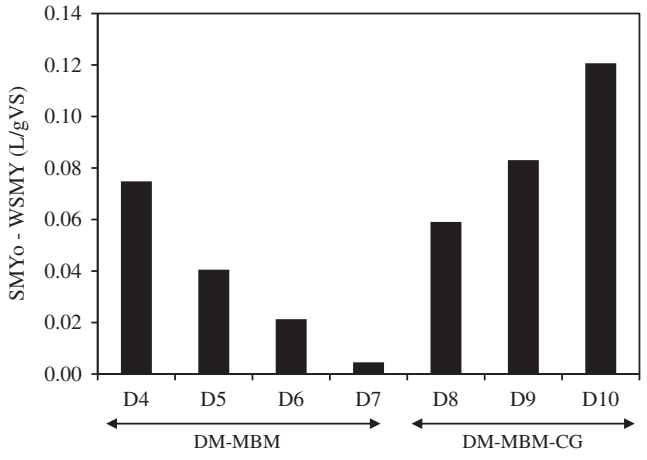

Fig. 4. Differential between specific methane yield (SMY_0) and weighted specific methane yield (*WSMY*) to show the synergistic effects of substrates during anaerobic co-digestion.

Table 6
Digestion parameters before and after anaerobic digestion.

		pH		Total VFA (mg/L)		HAc (mg/L)		HPr (mg/L)		n-HBu (mg/L)		HPr/HAc	
	Digester	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
Experiment I	D1	7.54	7.64	916.94	0.00	629.30	0.00	173.72	0.00	113.92	0.00	0.28	0.00
	D2	7.71	7.86	71.01	0.00	71.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	D3	8.01	7.76	29.75	23.39	29.75	23.39	0.00	0.00	0.00	0.00	0.00	0.00
Experiment II	D4	7.54	7.54	1201.33	0.00	738.47	0.00	162.02	0.00	257.78	0.00	0.22	0.00
	D5	7.56	7.87	1097.00	0.00	669.96	0.00	162.59	0.00	223.53	0.00	0.24	0.00
	D6	7.51	7.86	1127.40	0.00	679.60	0.00	181.02	0.00	216.11	0.00	0.27	0.00
	D7	7.45	7.90	1306.24	168.65	757.59	168.65	245.64	0.00	256.00	0.00	0.32	0.00
Experiment III	D8	7.57	7.80	970.34	34.22	653.57	34.22	194.92	0.00	121.84	0.00	0.30	0.00
	D9	7.60	7.79	933.30	24.34	635.51	24.34	182.74	0.00	115.04	0.00	0.29	0.00
	D10	7.64	7.77	881.21	31.93	604.41	31.93	167.00	0.00	109.80	0.00	0.28	0.00

when the differential is positive; otherwise antagonistic effect. As shown in Fig. 4, all treatments in the co-digested experiments showed synergistic effects. However, the differentials decreased significantly ($p < 0.05$) with the increase of MBM in experiment II, whereas they were increased significantly ($p < 0.05$) with the increase of CG in experiment III. At D4, the differential methane yield was 0.07 L/gVS and decreased by 43%, 71% and 86% in D5, D6 and D7, respectively. On the contrary, compared to D8, the differential methane yield was increased by 33% and 100% at D9 and D10, respectively, and the highest was 0.12 L/gVS at D10. The increase or decrease of differential methane yield can be attributed to different factors such as nutrient balance, buffer capacity, effect of toxic compounds, enzyme activities, and trace metals (Labatut et al., 2011; Li et al., 2011), which subsequently affect SMY_0. However, in this study, the decrease of differential methane yields against MBM concentration might be attributed to ammonia and VFA inhibition caused by a low C/N ratio, whereas the improvement of differential methane yield with CG concentration might be attributed to the increase of C/N ratio by CG addition. This is consistent with the report of Esposito et al. (2012) and Li et al. (Liu, 2013) that C/N ratio has an important influence on the synergistic effect of co-digested substrates. Therefore, using DM as base substrate, a ratio of MBM to CG of less than 1 (gVS MBM:gVS CG) is preferable for an efficient anaerobic co-digestion process. However, care must be taken to avoid the oversupply of CG that may cause an accumulation of intermediates and process failure.

Process stability

The pH, individual and total VFA before and after anaerobic digestion are shown in Table 6. In general, the final pH was in the range of a "healthy" anaerobic digestion process. However, a slight increase of pH was observed with the increase of MBM (experiment II), which may be attributed to ammonia accumulation that increases the pH in the digesters resulted from the formation of ammonium carbonate and the removal of carbon dioxide (Möller and Müller, 2012). On the other hand, the increase of CG (experiment III) led to a slight decrease of final pH, which probably resulted from VFA accumulation during the anaerobic co-digestion process. Although, the initial pH increased with CG as a result of high pH of CG (13.3) (Andriamanohiarisoamanana et al., 2016), it did not affect the final pH of the anaerobic co-digestion.

As given in Table 6, the three main volatile fatty acids; acetic acid (HAc), propionic acid (HPr) and n-butyric acid (n-HBu) were present in DM, while HAc was only present in MBM and CG before digestion. However, for the mixture substrates, all major VFAs were present before anaerobic co-digestion. The substrate that has the highest initial total VFA was DM followed by MBM. This is obvious as DM has already passed through fermentation process in cattle rumen. At the end of the digestion, the only VFA present was HAc at D3, D7 and the three digesters in experiment III, indicating that, generally the digestion process was successful. The residual HAc can be attributed to the length of experimental period which was not enough to completely convert all produced VFAs to methane and the long λ in those digesters. Despite the difficulties encountered by the anaerobic co-digestion of MBM and/or CG that caused lower hydrolysis rate constant or longer period for effective methane production, the process was stable and high methane yields were obtained.

Conclusions

Anaerobic co-digestion of meat and bone meal and crude glycerol with dairy manure is an alternative technology to recover energy from MBM and CG. Methane yield increased with the increase of MBM in anaerobic co-digestion of DM and MBM, while it increased together with CG in the anaerobic co-digestion of MBM and CG using DM as base substrate. In general, the process was stable. However, the anaerobic mono-digestion or co-digestion of CG showed residual amount of acetic acids. Similarly, at MBM of 20 g (D7), the highest acetic acid was observed. Hydrolysis rate constant and lag-phase increased along with CG content, implying that longer digestion period is required to have T_{90}. However, synergistic effect was increased with the addition of CG, while decreased with the increase of MBM content. Therefore, in anaerobic co-digestion, the duration of co-digestion process can be determined by the physico-chemical characteristics of the co-digested substrates, whereas, at the same organic loading rate, methane yield is dependent on the carbon to nitrogen ratio.

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