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Methane Production of *Pistia Stratiotes* as a Single Substrate and as a Co-Substrate with Dairy Cow Manure

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Abstract: Mono anaerobic digestion (AD) of dairy cow manure (DCM) is constrained by high moisture, ash and crude fibre content. Anaerobic co-digestion DCM and other biomass is one of the methods to overcome this drawback. This study aimed to evaluate: methane production from different parts of *Pistia stratiotes* (PS), methane production from the mixed substrate of PS and DCM in different proportions of PS in terms of volatile solids (VS) (0%, 7.99%, 14.91%, and 20.94%) using continuous digesters, and the potency of biogas yield from the digested slurry. Methane production from the whole plant, shoot system, and root of PS was 405.68, 416.82, and 326.42 L/kg VS, respectively. The highest methane production was obtained from the shoot system because that part contained higher crude protein and hemicellulose contents. Utilization of PS as a co-substrate for AD of the DCM can increase methane production by 28.65–56.98% compared to the control digester. No effect on pH, total ammonia nitrogen and total volatile fatty acid indicated that PS was suitable as a co-substrate of DCM and can significantly increase methane yield of the mixed substrate. AD of digested slurries showed that to recover the biogas production from the mixed substrate, the post-digestion treatment should be applied before the slurries are used as organic fertilizer.

Keywords: co-digestion; methane; manure; aquatic weed; post digestion

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1. Introduction

Imbalanced nutrient concentrations cause mono-digestion of animal manure to produce methane under sub-optimal conditions. Anaerobic digestion (AD) of livestock manure with a high protein content, such as pig and chicken manure, often results in too high concentrations of total ammonia nitrogen in the digester [1]. Meanwhile, in the case of AD of dairy cow manure (DCM), a low methane production per ton of substrate is obtained due to the high concentrations of moisture, ash and fibre fraction in the DCM [2]. Therefore, co-digestion of animal manure with other biomasses is proposed as an attractive strategy to improve methane production of livestock manure because it can result in balanced nutrient concentrations in the mixed substrates [2]. Currently, biomass that is used as anaerobic co-digestion with animal manure is largely fuelled by energy crops, where the increase in co-substrate demand will have an impact on increasing competition in food and feed supplies globally [3]. Therefore, the co-substrate biomasses for the AD of animal manures are better if they do not compete with the needs for food and animal feed. Thus, the aquatic weed *Pistia stratiotes* (PS) seems to fulfill that criterion.

Aquatic weed PS is known as one of the major invasive aquatic species. It can extensively cover the surface of ponds and lakes and can create various environmental problems due to its abundance and high reproducibility either in clear waters or wastewaters [4]. PS is known as one of the world's most dangerous invasive plants. This crop has great potential as a source of biomass and it is an easily available substrate due to its massive growth rate in natural bodies of water compared to food crops [5]. The biomass production

of PS is about 2.4 tons of dry biomass per hectare per year [6]. This feature along with the chemical composition of PS allows the further utilization of PS as a co-substrate with the DCM to enhance methane production of the mixed substrates through the AD process.

A study of methane production from PS has been conducted by [7–10] (Table 1). However, to the best of our knowledge, there is a lack of information regarding the study of the process performance of mesophilic biogas digester treating mixed substrate of PS and DCM at various ratios using a continuous feeding bio-reactor. Utilization of PS as a co-substrate with the DCM will increase organic matter and nutrient concentrations in the final substrate and it is proven to be able to increase methane production from the final substrate, which was a mixture of several substrates rather than that in the single substrate [11,12]. Since the retention time of substrates in the digester is limited, some organic materials and nutrients will remain in the digested slurries. Hence, the digested slurries still have the potential to be utilized as biogas feedstock. Therefore, the objectives of this study were (1) to evaluate the process performance of continuously stirred tank reactors (CSTR) with different proportions of PS in the final substrate, (2) to evaluate methane production from the different parts of PS, namely the whole plant, shoot system (i.e., the above-ground part), and roots of PS, and (3) to examine the biogas production of the digested slurries from the CSTR digesters. It was hypothesized that utilizing PS as a co-substrate with DCM increases the methane production of the mixed substrate without interrupting the performance of the biodigester.

Table 1. Previous study showing the anaerobic digestion of PS.

Substrate	Digester Type	Level of Combination	Duration of Study	Temperature Evaluation	Gas Production	Ref.
PS solely	Batch	-	30 d	29.5–37.5 °C	533–707 L biogas/kg VS	[7]
PS solely	Batch	-	30 d	37 °C	43 L CH_4/kg VS	[8]
Potato waste and PS	Batch	1:1	51 d	37 °C	447.4 L CH_4/kg VS	[9]
PS solely	Batch	-	62.5 d	37 °C	321 L biogas/kg VS	[10]

2. Materials and Methods

2.1. Experimental Set-Up

There were three experimental set-ups in this recent study: batch, continuous, and post-digestion tests. The first experimental study involved determining the ultimate methane yield of the whole plant, shoot system, and roots of PS. The second experimental study was to assess the process performance of four CSTRs operated at different ratios of DCM and PS, while the last study was to evaluate biogas production from digested slurry from each CSTR digester.

2.2. Batch and Post Digestion Test

Both experimental set-up batch and post-digestion tests were conducted using 500 mL infusion bottles as described by Møller et al. [13]. The experimental batch digesters contained a total of 200 g of inoculum and substrates with a ratio of inoculum: substrate of 1:1 in terms of volatile solid (VS). The substrates in this test varied between the whole plant, shoot system and roots of PS. The control batch digester solely contained inoculum so it was served to measure methane production from the inoculum. The net methane volume from substrates was obtained by subtracting the methane volume from the digester containing substrates and inoculum by the methane production from the inoculum.

The post-digestion test was also conducted using 500 mL infusion with a total of 200 g of digested slurry from each CSTR digester. The slurry was collected after the continuous feeding experiment was run for two hydraulic retention times (HRT). No inoculum was added in the post-digestion experiment. Each batch digester was closed using a butyl rubber stopper and it was sealed using an aluminium crimp. The headspace was flushed using nitrogen gas for two min. The batch and post-digestion test digesters

were maintained at 37 °C using incubators and they were run for 90 d and 30 d with three replications, respectively.

2.3. Continuous Experiment

In this section, four identical CSTRs (T1, T2, T3, and T4, see Table 2) with 7 L total capacity were operated with a working volume of 75% or 5.25 L and an HRT of 22 d. The digester was made of double-layer stainless steel. The mixing system was operated continuously using a propeller system and 36 revolutions per minute.

Table 2. Continuous experiment substrate properties.

	TS (%)	VS (%)	Crude Protein (%)	Proportion of PS (%)	pH	C/N Ratio
T1	6.43 ± 0.22	5.68 ± 0.36	0.59 ± 0.28	-	7.25 ± 0.16	33.43
T2	7.54 ± 0.15	6.24 ± 0.00	0.70 ± 0.16	7.99	7.15 ± 0.08	30.95
T3	8.64 ± 0.21	7.44 ± 0.57	0.80 ± 0.10	14.91	7.11 ± 0.08	32.29
T4	9.60 ± 0.15	7.90 ± 0.32	0.93 ± 0.23	20.94	7.10 ± 0.09	29.50

The experiment was started by filling all digesters with 5.25 kg of inoculum. On the second day, all digesters were fed 238.6 g of DCM after removing the same amount of digested slurries through a port at the base of the digesters. Each digester was fed DCM through a tube in which its outlet was submerged under the substrate level. This adaptation period was run for 21 d and after this period, the treatment period was started.

The treatments were the partial substitution of DCM with PS with the proportion of PS in the final substrate in terms of VS were 7.99%, 14.91%, and 20.94% in a term of VS for T2, T3, and T4, respectively, while T1 served as a control digester which was fed DCM solely. The CSTRs were maintained at 37 °C using incubators and this continuous experiment was run for three HRT or 66 d in total.

2.4. Inoculum and Substrate

The inoculum in this recent continuous study was digested slurry from a previous laboratory experiment [14]. The pH value, total solid (TS), and VS of the inoculum were 7.05, 4.05%, and 3.30%, respectively.

The inoculum for the batch experiment was the digested slurry of the continuous experiment. To minimize its gas production, it was kept anaerobically at 37 °C for two weeks. The digested slurry was then filtered out using a chiffon cloth and only the liquid fraction was then used to inoculate the substrates. The pH value, TS, and VS of inoculum for the batch digestion experiment were 7.39, 1.14%, and 0.56%, respectively.

The basal substrate was the DCM that was made by diluting dairy cow faeces with tap water with a ratio of 1:1.75 (w/w) to facilitate feeding to the digester. Dairy cow faeces were collected from the teaching farm at the Department of Animal Science, Faculty of Animal and Agricultural Sciences, Diponegoro University once a week. Once prepared, the DCM was stored in the refrigerator before being used to feed the continuous digester.

PS was collected from Swamp Pening at Salatiga, Central Java. It was washed using tap water and dried outdoors in the shade, followed by grinding using hammer milling and then screened to obtain a size of 1 mm. The PS powder was kept in sealed plastic at room temperature until used as a co-substrate with the DCM. The chemical composition of PS meal is presented in Table 3.

Table 3. Chemical composition of PS meal.

Component (%)	Shoot System	Root	Whole
Mass Balance (% of fresh)	72.25 ± 0.22	27.25 ± 0.22	100
Moisture	9.05 ± 0.20	11.04 ± 0.20	9.73 ± 1.37
Dry matter	90.95 ± 2.06	88.96 ± 2.06	90.27 ± 1.7
Volatile solids	65.90 ± 0.69	61.59 ± 0.69	65.36 ± 3.43
Ash	25.05 ± 0.17	27.66 ± 2.51	24.91 ± 4.64
Crude protein *	11.05	9.17	10.52
Extract ether	1.49 ± 0.07	1.40 ± 0.06	1.49 ± 0.07
Crude fibre	20.50 ± 1.33	21.14 ± 2.26	29.40 ± 0.59
Extract free nitrogen	32.86 ± 1.39	29.59 ± 2.20	23.95 ± 0.53
Neutral detergent fibre	75.47 ± 2.36	77.70 ± 0.69	78.41 ± 1.95
Acid detergent fibre	35.39 ± 0.63	46.09 ± 2.55	53.03 ± 3.37
Lignin	28.96 ± 0.57	26.31 ± 0.46	34.85 ± 0.62
Hemicellulose	40.09 ± 1.74	31.61 ± 3.24	25.38 ± 1.42
Cellulose	6.43 ± 1.20	19.78 ± 3.01	18.18 ± 3.98
C/N ratio	20.71	23.32	21.57

*: no replication during sample analysis.

2.5. Analytical Methods

Biogas production which was produced from batch test and continuous test digesters was directed to the 500 mL infusion bottle containing 4% NaOH (Merck®, cat no: 1064981000, Merck KGaA, Darmstadt, Germany) solution to absorb CO_2 [15] using a 5-mL Teflon tube. The NaOH solution was changed once a week. Methane production was collected using a Tedlar gas bag (Hedetech-Dupont, HedeTech Co., Ltd. Xigang district, Dalian, Liaoning, China) and it was measured daily for the continuous experiment and periodically for the batch experiment using the water displacement method as described by Sutaryo et al. [16].

Biogas production which was produced from the post-digestion test digesters was measured using the acidified water displacement method as described by Møller et al. [17]. The pH value was measured using a digital pH meter (Ohaus® ST300 pH meter, Ohaus Corporation, Parsippany, NJ, USA). TS of the sample was analysed by drying the sample at 105 °C for 7 h [18]. Ash concentration was determined by the combustion of the dried sample (TS) at 550 °C for 7 h [18]. VS concentration was measured by subtracting the TS value by the ash value. Extract ether was determined using the Soxhlet extraction method. Nitrogen (N) concentration was analysed using the Kjeldahl standard method, while total organic carbon (C) was measured by dividing the VS concentration value by 1.8, according to [19]. The C/N ratio was measured by dividing total organic carbon by total N. Acid detergent fibre (ADF), neutral detergent fibre (NDF), and lignin content of PS were analysed according to [20]. The concentration of hemicellulose in PS was calculated by subtracting the NDF concentration by the ADF concentration, while the concentration of cellulose in PS was calculated by subtracting the ADF concentration by the acid detergent lignin (ADL) concentration, and lignin concentration was assumed to be equal to the ADL [17]. Total volatile fatty acids (VFA) were measured using the steam distillation method. Total ammonia nitrogen (TAN) concentration was analysed using the distillation and titration method according to APHA [18]. The observed variables data were tabulated and statistically analysed using analysis of variance at the 5% confidence level according to Gomez and Gomez [21]. A Duncan multiple range test was applied in post analysis of variance.

3. Results

3.1. Batch Digestion Test

Methane production (in the unit of L/Kg VS) from different parts of PS is presented in Figure 1 and Table 4. The different parts of PS gave a significant effect ($p < 0.05$) on the methane yield for 30, 60, and 90 d of measurement. The highest methane production

resulted from the digestion of part of the shoot system. That phenomenon was caused by the different chemical compositions in each part (Table 3). The shoot system part contained higher protein and hemicellulose contents than those in the other parts (Table 2). According to Anukam et al. [22], the bioconversion of organic matter into biogas consists of four stages that must be in prime balance. The first step is hydrolysis in which the insoluble macro molecules are converted into soluble monomer organic compounds. The substrate containing better nutrient composition can produce a higher intermediate product for the next step and thereby can produce more methane production per ton of substrate.

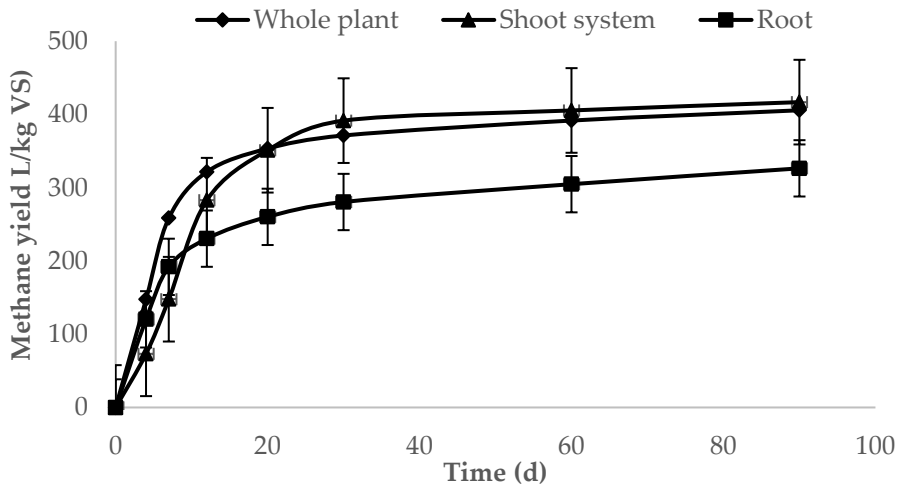

Figure 1. Methane production different part of *Pistia stratiotes*.

Table 4. Methane yields different part of *Pistia stratiotes*.

	Cumulative Methane Yield at 30 d (B_0: 30 d)	Cumulative Methane Yield at 60 d (B_0: 60 d)	Cumulative Methane Yield at 90 d (B_0: 90 d)
	(L/kg VS)		
Whole plant	371.29 ± 12.17 [a]	391.67 ± 17.52 [a]	405.68 ± 17.27 [a]
Shoot system	391.56 ± 17.13 [a]	405.45 ± 15.18 [a]	416.82 ± 18.80 [a]
Root	280.47 ± 4.74 [b]	304.81 ± 8.52 [b]	326.43 ± 10.80 [b]

The values in each column followed by a different superscript differ significantly ($p < 0.05$).

Methane production from whole PS in this study was higher than that in the study of Madenoğlu et al. [10], who reported that biogas production of whole PS after 63 d of incubation period at a temperature of 35 °C was 321 L/Kg VS with methane content of 72.5%. In both studies, a previous study [10] and this recent study, the PS sample was ground and then screened using a 2-mm screen size and a 1-mm screen size, respectively. This difference in physical size of the samples can probably explain why this recent study resulted in a higher methane production compared to the previous study of Madenoğlu et al. [10]. Particle size reduction can promote the AD of biomass by improving the accessibility of the biomass and increasing its susceptibility to microbial and enzyme attacks [23]. Despite the difference between the two results, the methane production data from PS confirm that PS has a high potential to produce biogas, one of the renewable energies, since the biomass production of PS is about 2.4 tons of dry biomass per hectare per year [6]. However, as an aquatic plant, in general, it has a high moisture content. In addition, in Table 3 it can be seen that PS meal has a high ash concentration. This is certainly a weakness of this biomass, considering that water and ash are not the main nutrients to support the microorganisms' activities. Li et al. [2] reported that high moisture and ash content lead to low methane production per tonne of sample.

3.2. Continuous Study

The trend of methane yield in the continuous study using CSTR digesters treating mixed substrates of DCM and PS at various proportions of PS in the mixed substrate (Table 2) is presented in Figure 2 and Table 5. Methane production (in the unit of L/kg substrate) from T2, T3, and T4 increased by 28.65%, 49.58%, and 56.98%, respectively, compared to that from T1. Utilization of PS as a co-substrate for AD of DCM gave a significant effect on methane production in all unit measurements (Table 5). A study from Orangun et al. [24] also showed the same phenomenon that co-digestion of goat manure (GM) with food waste (FW) can significantly increase the methane yield of the mixed substrate than that in the single substrate. The combination of GM and FW at some levels gave methane production from 2.2% to 107.6% higher than that in FW alone.

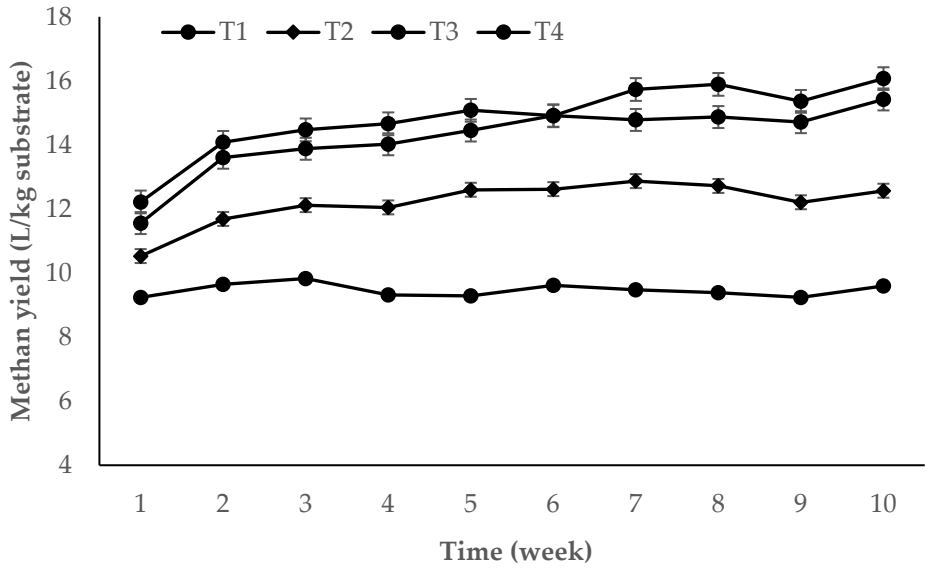

Figure 2. Trends of methane production in continuous study.

Table 5. Methane yield, TAN concentration, total VFA, VS reduction and pH value of digested bio-slurry.

	Methane Yield			TAN	Total VFA	VS Reduction	pH
	(L/kg Substrate)	(L/L Digester Volume)	(L/kg VS Added)	(mg/L)	(mM)	(%)	
T1	9.46 ± 0.47 ^{a}	0.43 ± 0.02 ^{a}	166.51 ± 8.24 ^{a}	72.60 ± 14.42	166 ± 23.19	33.42 ± 2.13 ^{a}	6.94 ± 0.12
T2	12.17 ± 0.95 ^{b}	0.55 ± 0.04 ^{b}	195.11 ± 15.23 ^{b}	75.40 ± 17.02	171 ± 21.32	35.26 ± 3.76 ^{ab}	6.99 ± 0.13
T3	14.15 ± 1.37 ^{c}	0.64 ± 0.06 ^{c}	190.16 ± 18.35 ^{bc}	75.40 ± 15.81	169 ± 28.85	37.32 ± 1.59 ^{bc}	7.03 ± 0.16
T4	14.85 ± 1.43 ^{d}	0.67 ± 0.06 ^{d}	187.99 ± 18.08 ^{cd}	79.60 ± 12.32	188 ± 19.32	38.87 ± 1.18 ^{c}	7.09 ± 0.12

Different superscripts in the same column are significantly different ($p < 0.05$)

Methane production from the T1 digester (control) treating the DCM solely was 166.51 L/Kg VS. A previous study of Dong et al. [25] found that methane yield from DCM using a plug flow biodigester with 25-d HRT and a temperature of 37–40 °C was in the range of 150–230 L/kg VS. Therefore, methane production of the control digester in this recent study was in line with the previous study. It can be seen in Table 1 that co-digestion of PS and DCM can increase VS and crude protein concentrations in the final substrate. TS concentration of animal manure is in the range of 7–9% [26]. Therefore, increasing the VS concentration of substrate can increase methane production per ton of substrate, since the availability of raw materials for biogas production by microorganisms also increases.

A higher nutrient concentration in the substrate can stimulate microorganisms' activities better during the bioconversion of organic matter into biogas. Gerardi [27] stated that bioconversion of organic materials in the waste into biogas is a multistage process

including hydrolysis, acidogenesis, acetogenesis, and methanogenesis. During hydrolysis, the macromolecules such as polysaccharide, protein and lipid are degraded into simple soluble nutrients (simple sugar, amino acids and fatty acids) by exoenzymes. Once solubilized, these simple nutrients enter the cell and are degraded by endoenzymes inside the microorganism. Anaerobe microorganisms obtain their energy for reproduction and cellular activity from the degradation of a relatively small number of simple substrates produces new bacterial cells and products. In the methanogenesis, the final stage of bioconversion, the majority of biogas is formed by the degradation of acetate into biogas by acetotrophic methanogens. Hence, in this recent study, methane production increased along with the accretion level of PS in each treatment. This fact was strengthened by the fact that VS reduction also increased significantly ($p < 0.05$) along with the additional proportion of PS in the mixed substrate (Table 3). Hagos et al. [28] reported that anaerobic co-digestion has numerous advantages such as improving the stability of the digestion process, reducing the concentration of inhibitors, balancing the nutrient needs, meeting the water requirements, reducing the greenhouse gas emissions into the atmosphere, increasing the synergistic effect between anaerobe microorganisms, increasing the VS concentration of the mixed substrate, and decreasing the operating cost. Chow et al. [29] stated that the advantages of anaerobic co-digestion are the supply of missing nutrients that are introduced by the co-substrates, strong potential to contribute to both pollution control and energy recovery, and ability to increase the efficiency of organic waste degradation. In addition, it can create safe and higher nutrient value of the digested slurry, and improved buffering capacity of the biogas digester [30]. In this study, a mixture of PS and DCM can also improve the C/N ratio of the final mixed substrate (Table 1). As reported by Mao et al. [31], the optimum C/N ratio of the substrate in the AD process is in the range of 25–30.

3.3. Variables in the Liquid Phase

There was no significant effect ($p > 0.05$) of the utilization of PS as a co-substrate for AD of the DCM on the pH value of digested slurry (Table 5). The pH value of all treatments was in the normal range for AD conditions. As reported by Mao et al. [31], the optimum pH in the AD process is in the range of 6.8–7.4. The pH value tended to increase along with the increasing proportion of PS in the mixed substrate. This phenomenon was associated with the increasing concentration of TAN. However, the TAN concentrations in all treatments were much below of inhibitory threshold as reported by Yenigün and Demirel [32], in which the inhibitory threshold of TAN for un-acclimated starter and under mesophilic temperature (35 °C) is around 1700–1800 mg/L. As explained previously, better nutrient compositions in the mixed substrates along with the increasing proportion of PS caused better activities of anaerobic microorganisms yielding higher methane production and VS reduction. There was no significant effect ($p > 0.05$) of co-digestion of DCM and PS on the total VFA concentration. It can be a good indicator that the acetoclastic methanogens microorganism can convert acetate, a majority component in VFA, into biogas well. Emebu et al. [33] stated that there are two main pathways of the methanogenesis process, namely (1) acetoclastic methanogenesis (conversion of $C_2H_4CO_2$ to CH_4 and CO_2, and (2) hydrogenotrophic methanogenesis (reaction between H_2 and CO_2 to result in methane).

3.4. Post Digestion Test

Utilization of PS as a co-substrate for anaerobic digestion of DCM in different ratios of DCM/PS gave a significant effect ($p < 0.05$) on the residual biogas production resulting from post-digestion of digested slurries (Table 6). This result indicated that there were the remaining organic matters in the digested slurry when the percentage level of PS in the final mixed substrate increased. Therefore, when PS is used as a co-substrate with DCM, the post-digestion process of digested slurry is needed before the digested slurry is used as an organic fertilizer. A study by Thygesen et al. [34] found that methane production from the solid fraction of digested slurry from eight biogas plants in Denmark was in the

range of 156–240 L/Kg VS. Uludag-Demirer and Demirer [35] stated that post-digestion of digested slurry gives a dual impact by reducing methane emission due to its storage and land application, and recovering methane of the digestates.

Table 6. Biogas production during post digestion test.

Biogas Yield (L/Kg Digested Slurry)	
T1	3.68 ± 0.19^{a}
T2	4.23 ± 0.19^{b}
T3	4.50 ± 0.86^{c}
T4	4.86 ± 0.04^{d}

Values in each column followed by the different superscript letter are significantly different ($p < 0.05$).

4. Conclusions

This study demonstrated that PS has great potential to produce renewable energy in the form of biogas, both as a single substrate and co-substrate with DCM. The experiment results showed that methane production of the whole plant, shoot system and root of PS were 405.68, 416.82 and 326.42 L/kg VS, respectively. Utilization of PS as a co-substrate of DCM can significantly increase methane production from the mixed substrate by 28.65%, 49.58%, and 56.98% compared to that from the DCM solely. There was no effect of the co-substrate of PS and DCM on pH value, TAN and VFA concentration. The pH value of all treatments was in the normal range for AD conditions, while the TAN concentrations in all treatments were much below the inhibitory threshold. It indicated that PS was suitable to increase methane production from the mixed substrates. However, to recover methane production from the mixed substrate, post-digestion should be applied before the digested slurry is used as an organic fertilizer.

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Conflicts of Interest: The authors declare no conflict of interest.

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