



Biogas Production from Hydrolysate Napier Grass by Co-Digestion with Slaughterhouse Wastewater using Anaerobic Mixed Cultures

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Abstracts

Biogas production from co-digestion of hydrolyzed napier grass and slaughterhouse wastewater using anaerobic mixed cultures was conducted. Factors influencing methane production was investigated, i.e., initial pH (6, 7, 8) and carbon-nitrogen (C/N) ratio. Optimum conditions were initial pH of 7 and C/N ratio of 3.42. Under these conditions, a methane production (MP), methane production rate (MPR) and methane yield (MY) of 299.69 ml CH₄/L, 0.52 ml CH₄/L h, and 39.76 ml CH₄/g-COD were obtained. Using the optimal conditions, MP, MPR and MY from co-digestion of hydrolyzed napier grass and slaughterhouse wastewater (299.69 ml CH₄/L, 0.52 ml CH₄/L h and 39.76 ml CH₄/g-COD) were 1.82, 1.79 and 2.11 times greater than that of the controls (without inoculum or self fermentation) (164.63 mL-CH₄/L, 0.29 mL-CH₄/L h and 18.76 ml CH₄/g-COD). The energy production from co-digestion of hydrolyzed napier grass and slaughterhouse wastewater was 11.99 kJ/L.

Keywords: *methane production, hydrolysate napier grass, slaughterhouse wastewater*

1. Introduction

In the past decade, anaerobic digestion had received increasing attention due to its use for converting waste into biogas. Biogas can be used to replace petroleum and fossil fuels (1). This process has several advantages, such as its potential in reducing a CO₂ and other greenhouse gas emissions and reducing the amount of biodegradable municipal waste sent to landfills (1-2).

Previously, biogas production throughout anaerobic digestion used a single feedstock such as cattle manure or municipal solid waste (3-4). However, the efficiency of anaerobic digestion processes using a single feedstock is often limited by insufficient amounts of waste for large-scale production. Additionally, utilization of single substrate had disadvantages such as unfavorable carbon-nitrogen (C/N) ratios, low pH, and

high concentration of ammonia in some substrates (2, 5–6). Therefore, co-digestion of mixed substrates for biogas production has recently gained increasing attention.

Napier grass is a complex material that is composed of cellulose, hemicellulose and lignin. Commonly, cellulose and hemicellulose primarily contain glucose and xylose, respectively. They can be fermented to produce renewable energy using several microbial processes. For example, the highest cumulative biogas production of 26.25 L was obtained using a ratio of napier grass and inoculum of 1:2 (7). Sawasdee and Pisutpaisal (8) reported that the maximum methane content and methane yield of 53% and 122.4 mlCH₄/g-TVS remove was obtained using napier grass as the substrate. Wen et al. (9) reported that the maximum methane yield of pretreated napier grass by the consortia MC1 was 259 ml/g-VS, which were 1.39 times greater than the values of the untreated controls (9). This study showed that the pretreatments method was capable of significantly enhancing methane yields from napier grass (9). Based on the previous research, the pretreatment method is necessary to decompose lignin for processing cellulose and hemicellulose. The most widely used pretreatment methods were categorized into three major: physical, chemical and biological, respectively. Acid hydrolysis is a well known method and effective tool for converting lignocellulosic materials into fermentable sugars (glucose, xylose) (10). Hydrolyzed napier grass has a high carbon content which is a good carbon source for anaerobic digestion. However, hydrolyzed napier grass lacks nitrogen, which is essential for microbial growth and metabolic

activities during anaerobic digestion. Therefore, a nitrogen source is needed to co-digest with hydrolyzed napier grass to achieve maximum methane production. In this research, slaughterhouse wastewater was used in a co-digestion with hydrolyzed napier grass to produce methane as it is a good nitrogen source.

Successful co-digestion of various wastes to produce methane has been reported (11–13). Wang et al. (11) reported that dairy manure co-digested with chicken manure and wheat straw showed better substrate digestion than using a single substrate. Wu et al. (12) revealed that co-digestion of swine manure with corn stalks at a C/N ratio of 20 give an 11 and 16 fold increase in cumulative biogas production and cumulative methane volume when compared to swine manure digested alone. Moreover, Hill et al. (13) showed that greater methane production from dairy manure was achieved when the C/N ratio was adjusted to 25:1 using glucose.

Based on previous findings, the C/N ratio can greatly impact the efficiency of methane production (11–13). The previous findings reported that the optimum C/N ratio for biogas production is between 20 and 30 (14). A low or high C/N ratio than the optimum range could result in an adverse effects on methane production process (14). If the C/N is too low, the process may be inhibited by accumulation of NH₃ produced from protein degradation (15–17). A greater C/N ratio than the optimum range may be inhibited by the lack of nitrogen sources for methanogenic bacteria growth (15–17). However, other environmental factors such initial pH and temperature also play an important role in

methane production during co-digestion process. A change in the initial pH could affect hydrolysis of organic matter during anaerobic digestion (18). Decreased initial pH results in an increase in hydrolysis of organic matter and is also favorable for organic nitrogen and phosphorus decomposition (18). Therefore, in order to obtain maximal conversion of organic matter, the pH of the process must be considered (19–21).

The aim of this work was to evaluate the effect of the C/N ratio and initial pH on methane production during co-digestion of hydrolyzed napier grass and slaughterhouse wastewater.

2. Materials and methods

2.1 Inoculum and Feestocks

Upflow anaerobic sludge blanket (UASB) granules obtained from a brewery wastewater treatment process (Khon Kaen Province, Thailand) was used as an inoculum. It was kept in a refrigerator at 4 °C before use. The total solid (TS) and volatile solids (VS) concentrations of UASB granules

were 0.19 ± 0.04 g-TS/g-dry weight and 0.18 ± 0.03 g-VS/g-dry weight.

Napier grass (*Pennisetum purpureum*) Pakchong 1 strain was obtained from Sriviroj Farm Public Company Limited, Khon Kaen Province, Thailand. Prior to use, napier grass was chopped into small pieces, air dried and milled in a blender. The size of blended napier grass was 0.30 x 0.30 mm. was then kept in plastic bags and stored at room temperature. The compositions of napier grass are shown in Table 1. Slaughterhouse wastewater (SW) was taken from the cesspit of slaughterhouse in Udon Thani Province, Thailand. Cesspit is used as a pretreatment unit to remove the debris from the slaughterhouse before the influent is sent to the wastewater treatment pond. The chemical characteristics of the SW are shown in Table 1. The SW was kept in the freezer at -20 °C until used. The frozen SW was thawed in a refrigerator at 4 °C and mixed before using it as a methane production medium.

Table 1. The compositions of napier grass, microwave- H_2SO_4 pretreated napier grass and slaughterhouse wastewater

Components	Napier grass	Slaughterhouse wastewater (SW)	Microwave- H_2SO_4 pretreatment napier grass	
			Solid	HG fraction
Carbon content (%)	49.93 ^a	1.26 ^b	ND	3.40 ^b
Nitrogen content (%)	2.02 ^a	1.35 ^b	ND	0.012 ^b
TS	0.86 ^c	0.21 ^d	ND	10.67 ^d
VS	0.86 ^c	0.16 ^d	ND	5.08 ^d
Total chemical oxygen demand (g-COD/L)	NA	4.05	NA	5.34
Lignin (%w/w)	32.04	NA	22.90	NA
Cellulose (%w/w)	34.25	NA	29.90	NA
Hemicellulose (%w/w)	17.36	NA	13	NA
Total sugar (g/L)	NA	NA	NA	6.36
Glucose (g/L)	NA	NA	NA	1.63
Xylose (g/L)	NA	NA	NA	0.95
Arabinose (g/L)	NA	NA	NA	0.19
Acetic acid (g/L)	NA	NA	NA	ND
Furfural (g/L)	NA	NA	NA	ND

HG: hydrolysate napier grass

NA: not applicable

ND: not detected

^a: unit in % w/w, ^b: unit in % w/v^c: unit in g/g-dry weight, ^d: unit in g/L

2.2 Pretreatment of napier grass

Microwave- H_2SO_4 pretreatment of napier grass was done in a LG/MS2022D microwave. The microwave- H_2SO_4 pretreatment conditions were set according to Khamtib et al. (22). Then a solid residue was separated by filtration through a thin layer of cloth. The pH of hydrolyzed napier grass (HG) was adjusted to 10 by addition of $Ca(OH)_2$ and the resulting precipitate was removed by centrifugation. HG was then reacidified to pH 7 with 2N HCl, followed

by centrifugation. The supernatant was collected and analyzed to determine its concentrations of sugars (glucose, xylose, arabinose) and inhibitors (furfural, acetic acid) using high performance liquid chromatography (HPLC). The chemical characteristics of HG are shown in Table 1.

2.3 Methane production from HG co-digested with SW using mixed anaerobic cultures

Methane production from co-digestion

of HG and SW was conducted in 120 ml serum bottles with a 85 ml working volume. Two factors, namely the initial pH (6, 7, 8), and C/N ratio (2.18, 3.42, 5.87) were selected for investigation. The methane production medium contained 7 g-VS/L inoculum, a basal salt medium (BA medium) for trace elements, and substrate at different C/N ratio (Table 2). The initial pH of the medium was adjusted to values of 6, 7 and 8 using either 1N NaOH or 1 N HCl. The serum bottles were capped with rubber stoppers and aluminum caps. The gas in the

headspace was flushed with nitrogen gas to create an anaerobic condition. The serum bottles were incubated at room temperature ($35 \pm 4^\circ\text{C}$). During the incubation, the volume of biogas was measured using wetted gas syringe's method (23). All treatments were conducted in triplicate. Methane production was continued until biogas generation ceased. A self fermentation was set up in a similar manner (under an optimal pH and C/N ratio) without inoculum.

Table 2. The chemical oxygen demand (COD) value and C/N ratio of mixed hydrolysate napeir grass and slaughterhouse wastewater

pH	HG/SW (%v/v)	tCOD (g/L)			C/N ratio
		initial	Final	Consumed	
6	0.5:1	11.60 \pm 2.14	4.12 \pm 3.48	7.54 \pm 2.13	2.18
	1:1	12.15 \pm 2.33	4.63 \pm 1.45	7.52 \pm 3.21	3.42
	2:1	13.84 \pm 7.86	4.84 \pm 1.98	9.00 \pm 4.12	5.87
7	0.5:1	10.16 \pm 4.32	2.34 \pm 1.87	9.81 \pm 2.76	2.18
	1:1	11.16 \pm 4.21	4.12 \pm 2.31	7.54 \pm 3.18	3.42
	2:1	12.89 \pm 5.11	4.75 \pm 2.46	9.09 \pm 4.12	5.87
8	0.5:1	11.20 \pm 5.12	3.59 \pm 1.21	8.56 \pm 4.44	2.18
	1:1	11.90 \pm 3.21	2.55 \pm 1.32	9.10 \pm 2.17	3.42
	2:1	13.35 \pm 7.12	5.33 \pm 3.21	8.51 \pm 5.41	5.87

2.4 Analytical methods

Biogas composition, including of methane, nitrogen, and carbon dioxide, was determined using a gas chromatograph (GC, Shimadzu 2014, Japan) equipped with a thermal conductivity detector (TCD) and a stainless steel column packed with shin charcoal carbon (50/80 mesh). The GC-TCD conditions were set according to Saraphirom and Reungsang (24). The volume of biogas produced was calculated

using a mass balance equation (25). pH was measured using a digital pH meter (Sartorius, Germany). Concentrations of TS and VS were measured using a 10 g sample at 105°C for 4 h and 550°C for 2 h, respectively. The concentration of sugars (glucose, xylose and arabinose), furfural and acetic acid in HG were analyzed using HPLC (Shimadzu LC-10AD) with an Aminex HPX-87H column following the method of Fangkum and Reungsang (26). The concen-

tration of cellulose, lignin and hemicellulose were determined using a method of Sluiter et al. (27).

Methane production was calculated from measurement of headspace gas composition. The total volume of methane produced during each time interval was determined by the method of Zheng and Yu (25). The volumetric methane production rate (MPR) (mL-CH₄/L h) was calculated as the cumulative methane production divided by fermentation time (h).

3. Results and discussion

3.1 Compositions of slaughterhouse wastewater, napier grass and pretreated napier grass

Table 1 shows the compositions of the SW, napier grass and microwave-H₂SO₄ pretreated napier grass used in this study. It is notable that napier grass had a higher carbon content than SW, while its nitrogen content was lower than the SW (Table 1). Napier grass is a good carbon source and SW is a good nitrogen source. In general, a biogas production requires a balance of carbon and nitrogen to enhance microbial growth and metabolic activity (11–13). Therefore, the combination of napier grass and SW can be a good substrate for biogas production.

Napier grass was pretreated using a microwave-H₂SO₄ pretreatment method (22). Initially, the napier grass consisted of (all in %w/w) 32.04% lignin, 34.25% cellulose, and 17.36% hemicellulose. After pretreatment, the fraction of lignin, cellulose, and hemicellulose was decreased to 22.9, 29.9 and 13.0% w/w, respectively. Obviously, the content of lignin hemicellulose and cellulose in napier grass drops when the microwave-H₂SO₄ pretreatment was performed. Low lignin, hemicellulose, and

cellulose contents after pretreatment showed that this method effectively removed amorphous parts of the lignocellulosic materials, i.e., lignin and hemicellulose (28, 29), and it also hydrolyzed some microcrystalline cellulose (28, 29). This can be attributed to the fact that pretreatment methods preferentially breaks down lignin and also hydrolyzed hemicellulose rather than crystalline cellulose fraction (28, 29). In this case, microwave radiation was heating polar molecules, which generates rapid heating to polar substances and no heating to low polar substances (30, 31). Water is a strong polar substance, whereas cellulose is low polar substances. This leads to the intense vibration of water molecule, the homogeneity heating and the temperature in certain zones within the sample being higher than the temperature around zones (30, 31), which resulted in the deconstruction of lignocellulose and the solubilization of hemicellulose. As can be seen in Table 1, in HG, sugars such as glucose, xylose, and arabinose were detected after the microwave-H₂SO₄ pretreatment. The detected sugars were future used as the substrate to produce methane through anaerobic digestion process. Acetic acid and furfural were not detected in napier grass after this pretreatment (Table 1). Acetic acid is a byproduct derived from the acetylation of hemicellulose and lignin (32). Furfural is a byproduct obtained from the degradation of xylan (32).

3.2 Effects of the initial pH and C/N ratio on methane production in batch experiments

The effect of initial pH and C/N ratio on methane production results are shown in Figure 1 and Table 3. Figure 1A–C revealed

that an increase in incubation time from 0 to 300 h resulted in a dramatically increase in the cumulative methane production. This might be due to the microorganisms adapt themselves to utilize the easily degradation part (such as sugar) of HG and SW for methane production. A further increase in the incubation time greater than 400 h resulted in an increase in cumulative methane production (Figure 1A–C). This disciplinary might be due to the microorganism utilize some hard degraded part in HG and SW for methane production. The results from table 3 showed that variation of initial pH and C/N ratio led to changes in MP, MPR and MY. The pH values after incubation in all experiments ranged from 6.98 to 8.06 (data not shown). Under mildly acidic conditions (an initial pH of 6.0), methane producing bacteria were not favored resulting in low MP and MPR (Figure 1 and Table 3). Neutral and mildly alkaline pH values (pH of 7.0 and 8.0), were suitable for growth and methane production resulting in higher MP and MPR values (Figure 1 and Table 3). Our results are consistent with a previous study which found that an initial pH between 6.5 and 8.5 was an optimal for methane production by anaerobic mixed cultures (33). At an optimal pH, methane producing bacteria exhibited good efficiency in degrading organic matter (33).

As shown in Table 3, MP, MPR, and MY varied over the range of 94.36 to 300.12 mL-CH₄/L, 0.18 to 0.54 mL-CH₄/L h and 11.09 to 39.76 mL-CH₄/g-COD respectively. Increasing the C/N ratio from 2.18 to 3.42 resulted in an increase in MP, MPR and MY. However, it decreased when the C/N ratio was greater than 3.42. Our results showed that maximal MP, MPR were obtained at

the C/N ratio of 3.42 (299.69 mL-CH₄/L, 0.52 mL-CH₄/L h), respectively. The MP and MPR obtained at the C/N ratio of 5.87 (300.12 mL-CH₄/L, 0.54 mL-CH₄/L h) were not significantly different from the results obtained at the C/N ratio of 3.42 (Table 3). However, the MY at C/N ratio of 3.42 (39.76 mL-CH₄/g-COD) were comparable high than the MY at the C/N ratio of 5.78 (33.03 mL-CH₄/g-COD). In addition the digestion time required at a C/N ratio of 3.42 was shorter than obtained at a C/N ratio of 5.87 (Figure 1). Therefore, it is reasonable that a C/N ratio of 3.42 best suited for methane production due to its short digestion period. The digestion period or digestion time is a key process design parameter that is selected to ensure that the microorganisms in the reactor have adequate time to grow and reproduce (34). At the same time it is important for economic viability to ensure that the digester is operated to obtain the maximum rate of gas production.

The maximum methane content (41%) obtained in this study was comparable to the methane content (42 %) reported by Kim and Kang (35) whom produced methane from algal biomass and food waste leachate by anaerobic seed sludge. However, our methane content was much lower than the methane content obtained from raw sludge and food waste leachate (62.2%) (35) and chicken manure and agricultural wastes (93%) (36), respectively (Table 4). Moreover, the maximum methane yield obtained in this study was much lower than the methane yield obtained from co-digestion of canned seafood wastewater and glycerol waste (37) and cheese whey (38) ice cream (39) and brewery wastewater (40). The discrepancy might be due to the low organic loading rate used in this study and

also the different types of substrate and operation temperature. Moreover, the low C/N ratio (3.42) than the optimum range may be inhibited by the lack of carbon

sources for methanogens and inhibited by accumulation of NH_3 produced from protein degradation (15–17).

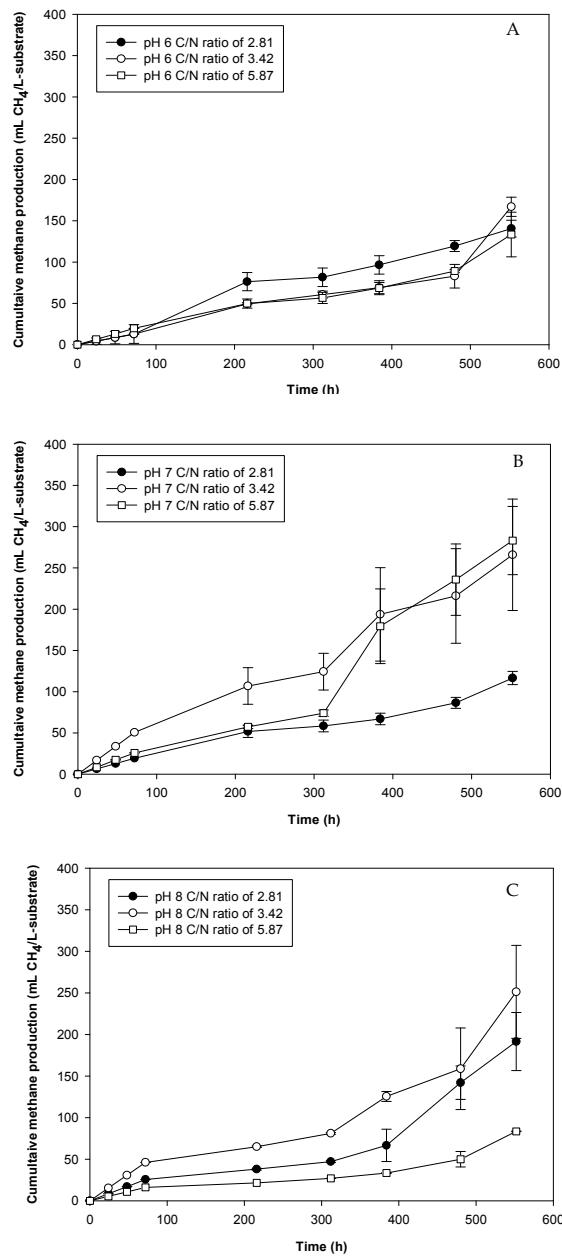


Figure 1. Cumulative methane production from hydrolysate napier grass by co-digestion with slaughterhouse wastewater at the various initial pH and C/N ratio (1A the initial pH of 6 and C/N ratio of 2.81, 3.42 and 5.87; 1B the initial pH of 7 and C/N ratio of 2.81, 3.42 and 5.87; 1C the initial pH of 8 and C/N ratio of 2.81, 3.42 and 5.87).

Table 3. Methane content, methane production, methane production rate and methane yield from co-digested of hydrolysate napier grass and slaughterhouse wastewater.

pH	C/N ratio	Methane content (%)	MP (ml CH ₄ /L-substrate)	MPR (ml CH ₄ /L-substrate h)	Methane yield (MY) (ml CH ₄ /g-tCOD)
6	2.18	18.49	149.16 ± 10.08	0.27 ± 0.01	19.79
	3.42	20.66	175.56 ± 2.69	0.32 ± 0.04	23.35
	5.78	15.66	146.35 ± 10.09	0.26 ± 0.03	16.26
7	2.18	13.15	129.52 ± 7.99	0.23 ± 0.01	13.20
	3.42	41.0	299.69 ± 6.79	0.52 ± 0.12	39.76
	5.78	39.41	300.12 ± 4.14	0.54 ± 0.07	33.03
8	2.18	26.42	208.53 ± 55.94	0.38 ± 0.10	24.36
	3.42	27.69	282.07 ± 34.90	0.52 ± 0.06	30.98
	5.78	10.40	94.36 ± 0.38	0.18 ± 0.01	11.09

The effects of inoculum addition on methane production from hydrolyzed napier grass by co-digestion with slaughterhouse wastewater were investigated. The control experiment consisted of hydrolyzed napier grass and slaughterhouse wastewater with no inoculation. Our results found that under optimal conditions, maximal MP, MPR and MY were 1.82, 1.79 and 2.11 times higher than its controls (without inoculum addition or self fermentation) (164.63 mL-CH₄/L, 0.29 mL-CH₄/L h and 18.76 ml CH₄/g-COD) (Figure 2). These results revealed

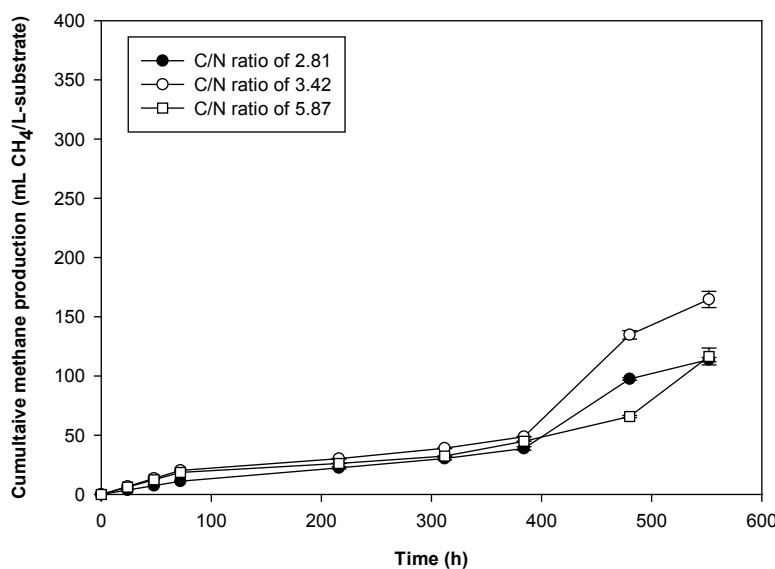
that an inoculum is needed for improved MP, MPR and MY.

3.3 Energy production from hydrolyzed napier grass co-digested with slaughterhouse wastewater

Energy productivity was determined based on methane production, density of methane (0.72 mg/ml) and its heating value (55.6 kJ/g). Under optimal conditions, maximal MP was 299.69 mL-CH₄/L. Therefore, energy production was [(299.69 x 0.72 x 55.6)] = 11.99 kJ/L.

Table 4. Comparison of methane content and methane yield from co-digested of hydrolysate napier grass and slaughterhouse wastewater with the literature research

Inoculum types	Type of substrate	Optimum conditions	Methane content (%)	Methane yield (ml CH ₄ /g-COD)	References
Anaerobic seed sludge	Algal biomass and food waste leachate	Ratio 1:1 (w/w), 35 °C, 120 rpm, and pH 7.5	42.2	-	(35)
		Ratio 1:1 (w/w), 35 °C, 120 rpm, and pH 7.6	62.2	-	(35)
Anaerobic seed sludge	Chicken manure (FCM) and agricultural wastes (AWS)	FCM: AWS ratio of 7:3 (v/v), 55 °C	93.0	-	(36)
Granule sludge	Canned seafood wastewater (CSW) and glycerol waste (GW)	99% CSW and 1% GW 36 °C pH range 6.9-7.2	-	309	(37)
Seed sludge	Brewery wastewater	8.3 kg COD/m ³ day HRT of 4.9 day	67-79	280-350	(38)
Seed sludge	Cheese whey	13 kg COD/m ³ day HRT of 4.9 day	-	340	(39)
Sludge	Ice cream	6 kg COD/m ³ day HRT of 0.5 day	-	320-340	(40)
USAB granules	Hydrolysate napier grass (HG) and slaughterhouse wastewater (SW)	HG: SW ratio of 1:1 (v/v) pH 7, 35 °C	40.9	39.76	This study
Self fermentation			19.5	18.87	This study

**Figure 2.** Cumulative methane production from hydrolysate napier grass by co-digestion with slaughterhouse wastewater at the initial pH of 7 without inoculum addition.

4. Conclusions

These results demonstrated that initial pH, as well as a ratio of hydrolyzed napier grass and slaughterhouse wastewater had an effect on MP and MPR. Optimal conditions for maximal MP and MPR were a C/N ratio of 3.42, and an initial pH of 7. Under optimal conditions, a maximum MP and MPR of 299.69 mL-CH₄/L and 0.52 mL-CH₄/L h were respectively achieved. This was 1.82 and 1.79 times higher than in the control experiment (without inoculum) (164.63 mL-CH₄/L and 0.29 mL-CH₄/L h), indicating a significant enhancement in MP and MPR by use of a seed inoculum.

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