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Co-digestion of sugarcane bagasse, microalgal biomass and cow dung for biohydrogen and methane production

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Abstract

This study investigated the use of untreated sugarcane bagasse (SCB), the biomass of a *Chlorella* sp., and cow dung (CD), as feedstocks for biohydrogen and methane production employing vermihumus as an inoculum. D-optimal mixture design was used to optimize the proportion of each feedstock for a single-stage anaerobic digestion (AD), and two-stage dark fermentation (DF) followed by AD to produce methane as well as biohydrogen and methane, respectively. Using a single-stage AD, a methane yield of 230 mL-CH_4/g-volatile-solids (VS), equivalent to an energy yield of 8.2 kJ/g-VS, was attained under the optimal conditions of 29.5 g VS/L of SCB, 23.9 g-VS/L of *Chlorella* sp. biomass, and 6.6 g-VS/L of CD. DF conducted as the first stage of the two-stage process yielded 24.41 mL-H_2/g-VS, under the optimal conditions of 16.3 g-VS/L of SCB, 41.7 g-VS/L of *Chlorella* sp. biomass, and 2.0 g-VS/L of CD. Further use of the hydrogenic effluent in AD yielded 140.17 mL CH_4/g-VS, leading to a total energy yield of 5.3 kJ/g-VS. Study results revealed that the single-stage AD process was effective in recovering energy (in the form of methane) from the feedstocks despite using no biomass pretreatment. They also showed that vermihumus could be used as an inoculum. The results also revealed the potential of the two-stage process for the production biohythane (a blend of biohydrogen and methane), a gas mixture that has better fuel properties than methane.

Keywords: D-optimal mixture design, Co-digestion, Renewable energy, Dark fermentation, Methanogenesis, Vermihumus

1. Introduction

The problems of global warming, climate change and air pollution resulting from the consumption of fossil fuels are the main driving forces behind the current vigorous research on alternative energy resources [1]. Biohydrogen and methane are among the renewable energy resources that have gained much attention as promising substitutes for fossil fuels. Hydrogen has a high energy content, 122 kJ/g. Additionally, its combustion yields only water as a by-product, making it very environmentally friendly [2]. Although methane has a lower energy content, 55.7 kJ/g, its combustion emits fewer greenhouse gases, compared with petroleum-based fuels. Methane can be used in various applications, including direct use in gas turbines for electricity generation, as a transportation fuel as well as a cooking and heating fuel [3].

Biohydrogen and methane can be produced *via* fermentation of biomass, using some of the first, second, or third generation processes. Second generation biomass, e.g., sugarcane bagasse (SCB) and cow dung (CD), are recognized as promising substrates. This is due to their ready availability at low cost, high carbohydrate content, and non-food nature [4]. SCB and CD have been reported to contain large cellulose fractions (33.78% and 15.30%, respectively) [5,6] that can be hydrolyzed to fermentable sugars for subsequent fermentation processes. Additionally, CD has been reported to contain trace elements, such as iron, nickel, and zinc [7] that are required for microbial activities during fermentation [8]. However, despite their high potential, the utilization of SCB and

CD is impeded by their imbalanced carbon to nitrogen (C/N) ratios. SCB and CD have C/N ratios of 26.0-160.0 and 7.0-23.8 [9-11], respectively. Their use could result in limited availability of nitrogen in a fermentation system. This would, in turn, result in slow microbial growth and low product formation. In this regard, a nitrogen-rich feedstock could be mixed with SCB and CD to adjust the C/N ratio so that it is favorable for microbial activity. Microalgal biomass has a high protein content and low C/N ratio [12]. This third generation feedstock could be a feasible co-substrate for fermentation with SCB and CD to improve the C/N ratio of such processes.

The current study aims to develop simple and cost-effective processes for biohydrogen and methane production. It investigates the use of SCB, CD, and the microalga biomass of a *Chlorella* sp. as fermentation feedstocks, employing vermihumus as an inoculum. Vermihumus, also known as vermicompost, is a product of a bio-oxidative process of organic matter facilitated by microorganisms present in the gut of earthworms, mainly the *Californian* Red Earthworm (*Eisenia fetida*) [13]. Vermihumus is a nutrient-enriched inoculum containing several minerals (e.g., iron, nickel, and zinc) and lytic enzymes (e.g., cellulase, protease, chitinase, and peroxidase) [14,15] that are beneficial for biohydrogen and methane production. It is also enriched with microorganisms, including an active microbial population of Clostridia and methanogenic archaea [16]. According to Pathma and Sakthivel (2012) [15], vermicomposting is a time- and cost-effective, as well as an environmentally friendly process. Therefore, this method could potentially be used for the production of low-cost inocula for use in biohydrogen and methane production. In the present study, a single-stage anaerobic digestion (AD) and a two-stage process consisting of dark fermentation (DF) followed by AD were employed to produce methane, along with biohydrogen and methane, respectively. The proportion of each feedstock was optimized using D-optimal mixture design software to attain the maximum yield of gaseous products. Energy yields from each process were determined to demonstrate the applicability of SCB, CD, and *Chlorella* sp. biomass as feedstocks, as well as vermihumus as an inoculum for biohydrogen and methane production.

2. Materials and methods

2.1 Inoculum and feedstocks

Vermihumus purchased from a local shop at Khon Kaen University, Khon Kaen, Thailand, was used as an inoculum for biohydrogen and methane production. It was sieved through a 0.5-mm screen and heated at 105°C for 3 h to inactivate methanogens before use as hydrogen inoculum. For methane production, the vermihumus was sieved and used without any pretreatment. The vermihumus (both heat-treated and untreated) was stored in air-tight plastic bags at room temperature until use.

SCB was obtained from a local sugar manufacturing plant (United Farmer & Industry Co., Ltd. (Phu Wiang Branch), Khon Kaen, Thailand). It was air dried, milled, sieved through a 0.5-mm screen, and then stored at room temperature in air-tight plastic bags for later use. CD was obtained from a cattle farm at the Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. It was dried at 105°C in a hot air oven, then ground using a kitchen blender. The ground CD was sieved through a 0.5-mm screen and stored in air-tight plastic bags at room temperature. *Chlorella* sp. biomass was purchased from Yantai Hearol Biotechnology Co. Ltd., Chengmai, Hainan, China. The biomass was obtained in the form of a dry powder. It was kept in air-tight plastic bags at -20°C for later use.

2.2 Experimental design

D-optimal mixture design was used to determine the optimum concentrations of SCB (X_1), *Chlorella* sp. biomass (X_2), and CD (X_3) for single-stage methane and two-stage biohydrogen and methane production processes. The concentrations of SCB, *Chlorella* sp. biomass, and CD were varied over the ranges 5-40 g-VS/L, 10-50 g-VS/L, and 2-20 g-VS/L, respectively. Using Design Expert software (Demo Version 7.0, Stat-Ease, Inc., Minneapolis, MN, USA), a total of 16 experimental runs were designed, with the total VS of each run set at 60 g-VS/L (Table 1).

A cubic model, Equation (1), was used to fit the results of biohydrogen and methane production, where Y is biohydrogen yield (HY) or methane yield (MY). β_i, β_{ij}, β_{ijk} are linear, quadratic, and cubic coefficients, respectively. δ_{ij} is a parameter of the model. The $\beta_i x_i$ term represents the linear mixing proportions and β_{ij} represents a synergistic or antagonistic effects of the proportions.

$$Y = \sum_{i=1}^{p} \beta_i x_i + \sum \sum_{i<j}^{p} \beta_{ij} x_i x_j + \sum \sum_{i<j}^{p} \delta_{ij} x_i x_j \left(x_j - x_i\right) + \sum \sum \sum_{i<j<k}^{p} \beta_{ijk} x_i x_j x_k \quad (1)$$

Table 1 Experimental design defining the proportions of SCB, *Chlorella* sp. biomass, and CD.

Run	Experimental factors		
	SCB (X_1, g-VS/L)	*Chlorella* sp. biomass (X_2, g-VS/L)	CD (X_3, g-VS/L)
1	5.27	34.73	20.00
2	22.23	35.77	2.00
3	5.56	50.00	4.44
4	30.53	27.47	2.00
5	30.33	19.39	10.28
6	21.27	27.37	11.36
7	5.56	50.00	4.44
8	19.96	20.04	20.00
9	40.00	16.75	3.25
10	5.01	44.30	10.69
11	22.23	35.77	2.00
12	28.25	11.75	20.00
13	28.25	11.75	20.00
14	5.27	34.73	20.00
15	11.59	39.26	9.15
16	40.00	16.75	3.25

2.3 Single-stage anaerobic digestion process

Using the conditions given in Table 1, a single-stage AD was carried out employing a feedstock to inoculum (F/I) ratio of 3 to 1 on a VS basis. SCB, *Chlorella* sp. biomass, and CD at the designated concentrations were transferred into 120-mL serum bottles. Then, basic anaerobic (BA) medium [17] was added to the bottles to make up a total volume to 70 mL in each bottle. The initial pH of the mixture was adjusted to pH 7.5 using 5 M HCl or 5 M NaOH, as appropriate, before the bottles were tightly capped and purged with nitrogen gas for 5 min to create anaerobic conditions. Incubation was performed at room temperature (35 ± 2°C) on an orbital shaker at 150 rpm for 95 days. Gas samples were collected at regular time intervals for gas composition analysis using gas chromatography (GC).

2.4 Two-stage dark fermentation and anaerobic digestion processes

Dark fermentation was conducted by transferring SCB, *Chlorella* sp. biomass, and CD to 120-mL serum bottles according to the experimental design (Table 1). Then, heat-treated vermihumus was added into bottles at a F/I ratio of 3 to 1 on a VS basis. A modified Endo nutrient solution [18] was subsequently added to each of the bottles to make up their volumes to 70 mL. Initial pH of the mixtures was adjusted to pH 5.5 using either 5 M HCl or 5 M NaOH, as appropriate. The bottles were tightly capped and flushed with nitrogen gas for 5 min to create anaerobic conditions. Incubation was carried out at room temperature (35 ± 2°C) on an orbital shaker at 150 rpm. Gas samples were collected periodically and analyzed using GC. When the production of biohydrogen ceased, the serum bottles were uncapped and the pH of the hydrogenic effluent was adjusted to 7.5 using 5 M NaOH. Then, untreated vermihumus was added into the bottles at a F/I ratio of 3 to 1 on a VS basis. The bottles were then capped and flushed with nitrogen gas for 5 min and further incubated at room temperature for 100 days for methane production.

2.5 Analytical methods

Total solids (TS) and VS of the feedstocks and hydrogenic effluents were determined using standard methods [19]. pH was measured using a pH meter (pH 500 Clean, USA). Biogas compositions were analyzed using GC (GC 2014, Shimadzu, Japan) following the method of Pattra *et al*. (2008) [20]. The GC was equipped with a

thermal conductivity detector and a 2-m stainless steel column, packed with Shin carbon (50/80 mesh). Results of biohydrogen and methane production were fitted with the modified Gompertz model, Equation (2), to obtain the kinetic parameters of the processes. Energy yield was calculated using Equation (3).

$$P = P_{max}\, exp\left(-exp\left(\frac{R_{max}e}{P_{max}}\,(\lambda - t) + 1\right)\right) \quad (2)$$

$$Y_{Energy} = \frac{Yield}{22{,}400} \times HV \quad (3)$$

where P is the predicted value of biohydrogen or methane yield (mL/g-VS), P_{max} is the maximum biohydrogen or methane yield (mL/g-VS), R_{max} is the maximum rate of gas production (mL/(g-VS·h)), λ is the lag time (h), Y_{Energy} is the energy yield (kJ/g-VS), *Yield* is the biohydrogen or methane yield (mL/g-VS), 22,400 is the molar volume of gas (mL/mol), and *HV* is the heating value of biohydrogen (242 kJ/mol) or methane (801 kJ/mol).

3. Results

3.1 Single-stage AD process for methane production

The production of methane differed with varying proportions of SCB, *Chlorella* sp. biomass, and CD as shown in Table 2. The methane production results were used to generate a cubic equation, Equation (4), which describes methane production under the conditions tested. One-way analysis of variance (ANOVA) of Equation (4) showed that the model was significant at a 95% confidence level (F-value = 7.5126, p-value = 0.0117). The coefficient of determination (R^2) and adjusted R^2 of Equation (4) were 0.9185 and 0.7962, respectively. However, the lack of fit of the equation was significant at a p-value = 0.0053 (Table 3).

Table 2 Methane yields attained using various proportions of SCB, *Chlorella* sp. biomass, and CD, through a single-stage AD process.

Run	Experimental factors			MY (mL-CH_4/g-VS)	
	SCB (X_1)	*Chlorella* sp. biomass (X_2)	CD (X_3)	Observed results[a]	Predicted results
1	5.27	34.73	20.00	188.94 ± 5.18	189.01
2	22.23	35.77	2.00	171.67 ± 2.81	169.21
3	5.56	50.00	4.44	153.34 ± 4.99	158.18
4	30.53	27.47	2.00	195.41 ± 7.86	201.24
5	30.33	19.39	10.28	236.74 ± 2.29	226.69
6	21.27	27.37	11.36	188.29 ± 2.41	203.59
7	5.56	50.00	4.44	158.43 ± 2.06	158.18
8	19.96	20.04	20.00	230.73 ± 0.81	221.11
9	40.00	16.75	3.25	173.06 ± 4.53	173.06
10	5.01	44.30	10.69	155.73 ± 1.55	147.85
11	22.23	35.77	2.00	174.72 ± 8.13	169.21
12	28.25	11.75	20.00	190.54 ± 4.10	200.69
13	28.25	11.75	20.00	205.24 ± 5.75	200.69
14	5.27	34.73	20.00	185.30 ± 4.11	189.01
15	11.59	39.26	9.15	165.55 ± 5.17	166.04
16	40.00	16.75	3.25	172.97 ± 8.01	173.06

[a] Calculated from triplicate samples

$$MY = -10.26X_1 + 2.14X_2 + 244.69X_3 + 0.42X_1X_2 - 7.00X_1X_3 - 7.18X_2X_3 + 0.12X_1X_2X_3 + 7.96X_1X_2\,(X_1 - X_2) + 0.007X_1X_3\,(X_1 - X_3) + 0.06X_2X_3\,(X_2 - X_3) \quad (4)$$

Table 3 ANOVA for the cubic model, Equation (4), describing methane production under the tested conditions.

Source	Sum of Squares	df	Mean Square	*F*-value	*p*-value
Model	8124.269	9	902.6966	7.5126	0.0117
Linear Mixture	4622.599	2	2311.299	19.2355	0.0025
X_1X_2	613.0243	1	613.0243	5.1018	0.0647
X_1X_3	166.6061	1	166.6061	1.3866	0.2836
X_2X_3	171.5396	1	171.5396	1.4276	0.2772
$X_1X_2X_3$	183.6897	1	183.6897	1.5287	0.2625
X_1X_2 (X_1-X_2)	1046.03	1	1046.03	8.7055	0.0256
X_1X_3 (X_1-X_3)	206.7671	1	206.7671	1.7208	0.2375
X_2X_3 (X_2-X_3)	148.3312	1	148.3312	1.2345	0.3091
Residual	720.9471	6	120.1578		
Lack of Fit	588.6897	1	588.6897	22.2555	0.0053
Pure Error	132.2573	5	26.45147		
Cor Total	8845.216	15			
R^2	0.9185				
Adjusted R^2	0.7962				

Based on the cubic model, Equation (4), numerical optimization revealed that a maximal MY of 250.1 mL-CH_4/g-VS could be obtained using 29.5 g-VS/L of SCB, 23.9 g-VS/L of *Chlorella* sp. biomass and 6.6 g-VS/L of CD (Figure 1). A confirmation experiment was conducted using the predicted optimal conditions. This yielded a MY of 229.47 mL/g-VS, which was within the 95% prediction interval (95% PI) of 186.89 to 295.77 mL/g-VS, confirming that the predicted conditions were valid and applicable. Fitting the results of the confirmation experiment with the modified Gompertz model, Equation (2), revealed that the lag time (λ) for methane production was 15.8 days, while the maximum methane yield (P_{max}) and methane productivity (R_m) were 226.7 mL/g-VS and 5.7 mL/(g-VS·h), respectively (Figure 2).

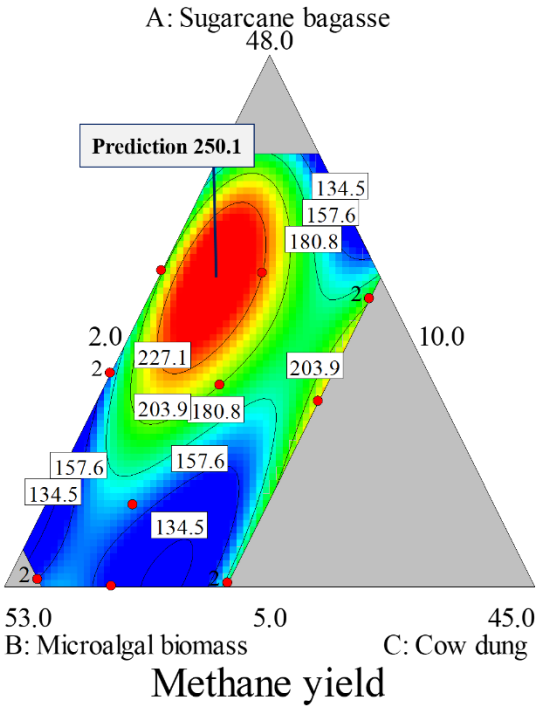

Figure 1 Contour plot showing the effects of SCB, *Chlorella* sp. biomass, and CD concentrations on methane yield under a single-stage AD process.

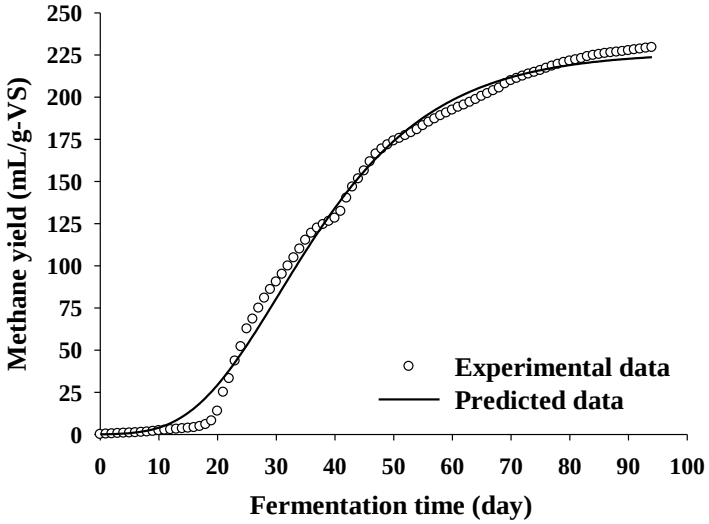

Figure 2 Methane production from mixed substrates of SCB, *Chlorella* sp. biomass, and CD under optimal conditions. The solid curve represents data predicted by the modified Gompertz model, Equation (2).

3.2 Two-stage process for biohydrogen and methane production

Table 4 shows that the production of biohydrogen differed with variation of the proportions of SCB, *Chlorella* sp. biomass, and CD. The results were best described by the cubic equation shown in Equation (5). ANOVA of Equation (5) revealed that the model was significant with an *F*-value and *p*-value of 413.01 and < 0.0001, respectively (Table 5). The lack of fit of the model (*p*-value = 0.3943), R^2 (0.9984) and adjusted R^2 (0.9960), confirmed that the model was highly applicable in predicting biohydrogen production under the conditions tested.

Table 4 Biohydrogen yields attained using various proportions of SCB, *Chlorella* sp. biomass, and CD, through dark fermentation.

Run	Experimental factors			HY (mL-H_2/g-VS)	
	SCB (X_1)	*Chlorella* sp. biomass (X_2)	CD (X_3)	Observed results[a]	Predicted results
1	5.27	34.73	20.00	26.76 ± 1.2	26.48
2	22.23	35.77	2.00	29.39 ± 0.8	29.22
3	5.56	50.00	4.44	30.73 ± 1.1	30.48
4	30.53	27.47	2.00	20.08 ± 0.6	19.99
5	30.33	19.39	10.28	16.49 ± 1.1	16.64
6	21.27	27.37	11.36	20.69 ± 0.2	20.47
7	5.56	50.00	4.44	30.30 ± 0.7	30.48
8	19.96	20.04	20.00	17.94 ± 1.0	18.08
9	40.00	16.75	3.25	26.53 ± 3.3	26.28
10	5.01	44.30	10.69	22.80 ± 0.5	22.92
11	22.23	35.77	2.00	28.93 ± 0.0	29.22
12	28.25	11.75	20.00	12.67 ± 0.1	13.00
13	28.25	11.75	20.00	13.42 ± 0.6	13.00
14	5.27	34.73	20.00	26.26 ± 0.6	26.48
15	11.59	39.26	9.15	26.85 ± 0.2	26.84
16	40.00	16.75	3.25	26.03 ± 0.6	26.28

[a] Calculated from five replicate samples

$$HY = 2.88X_1 + 0.65X_2 - 17.03X_3 - 0.09X_1X_2 + 0.41X_1X_3 + 0.54X_2X_3 - 0.007X_1X_2X_3 + 0.02X_1X_2(X_1 - X_2) - 0.004X_1X_3(X_1 - X_3) - 0.006X_2X_3(X_2 - X_3) \quad (5)$$

Table 5 ANOVA for the cubic model, Equation (5), describing biohydrogen production under the conditions tested.

Source	Sum of squares	df	Mean square	*F*-Value	*p*-value Prob > F
Model	526.06	9	58.45	413.01	< 0.0001
Linear Mixture	361.16	2	180.58	1275.96	< 0.0001
X_1X_2	22.29	1	22.29	157.48	< 0.0001
X_1X_3	0.54	1	0.54	3.8	0.0992
X_2X_3	0.83	1	0.83	5.84	0.052
$X_1X_2X_3$	0.57	1	0.57	4.03	0.0915
X_1X_2 (X_1-X_2)	51.54	1	51.54	364.17	< 0.0001
X_1X_3 (X_1-X_3)	0.81	1	0.81	5.74	0.0536
X_2X_3 (X_2-X_3)	1.42	1	1.42	10.03	0.0194
Residual	0.85	6	0.14		
Lack of Fit	0.13	1	0.13	0.87	0.3943
Pure Error	0.72	5	0.14		
Cor Total	526.91	15			
R^2	0.9984				
Adjusted R^2	0.9960				

Based on Equation (5), numerical optimization revealed that a maximal HY of 37.8 mL/g-VS could be achieved using 16.3 g-VS/L of SCB, 41.7 g-VS/L of *Chlorella* sp. biomass, and 2.0 g-VS/L of CD, as seen in Figure 3. Using the predicted optimal conditions, a confirmation experiment yielded a HY of 24.41 mL/g-VS.

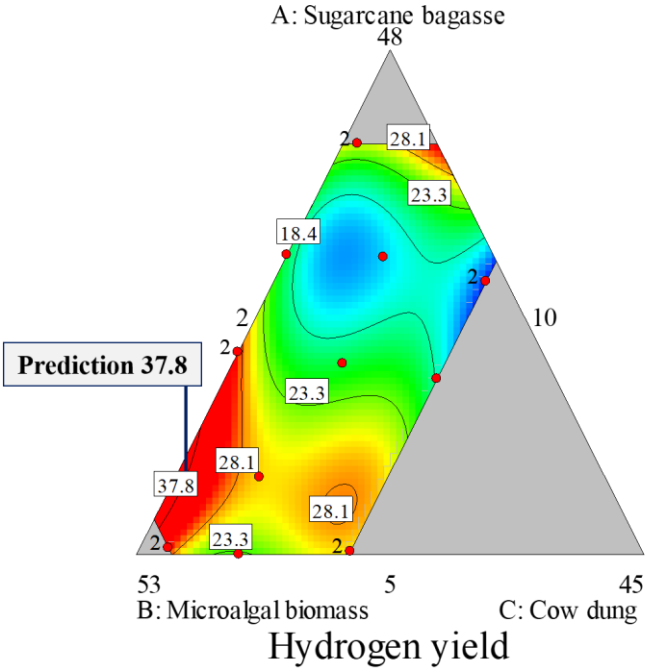

Figure 3 Contour plot showing the effects of SCB, *Chlorella* sp. biomass, and CD concentrations on biohydrogen yield under dark fermentation.

The effluent obtained after DF, also called hydrogenic effluent, was subsequently used as a feedstock for methane production in the second stage. The production of methane started soon after inoculation using untreated vermihumus, indicating that methanogens present in the vermihumus were active. After a short lag time, the production of methane increased sharply until 40 days into the process. This was followed by a more gradual increase in methane production to 140.76 mL/g-VS at 100 days (Figure 4). Modeling the experimental results

using the modified Gompertz equation (Equation (2)) revealed that the lag time (λ) for methane production was approximately 6 days, while the maximum methane yield (P_{max}) and methane productivity (R_{max}) were 141.3 mL/g-VS and 4.5 mL/(g-VS·h), respectively (Figure 4).

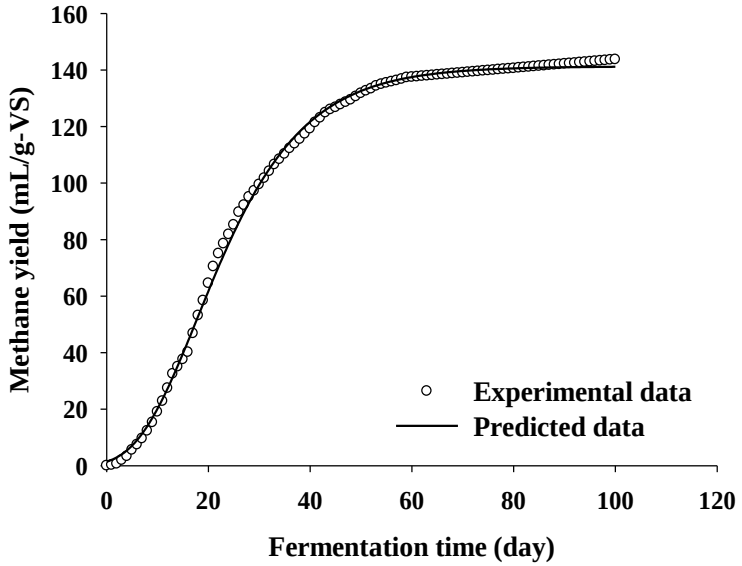

Figure 4 Methane production from a hydrogenic effluent obtained after dark fermentation under optimal conditions. The solid curve represents the data predicted using the modified Gompertz equation, Equation (2).

4. Discussion

The present study showed that biohydrogen and methane can be successfully produced by co-digestion of SCB, *Chlorella* sp. biomass, and CD, with no pretreatment of the feedstocks. Optimal conditions for a single-stage methane production were 29.5 g-VS/L of SCB, 23.9 g-VS/L of *Chlorella* sp. biomass, and 6.6 g-VS/L of CD, which yielded 250 mL-CH_4/g-VS, according to the prediction. Based on Equation (4), deviation from these optimum levels, *e.g.* increases in the concentration of SCB and decreases in the concentration of *Chlorella* sp. biomass, reduced the methane production. Since SCB is carbon-rich (high C/N ratio) [21] and *Chlorella* sp. biomass is protein-rich (low C/N ratio) [12], changes in their concentrations will directly affect the C/N ratio of the fermentation medium, which in turn affects methane production [22]. The recalcitrant cell walls of *Chlorella* sp. could potentially limit access to nitrogen and other nutrients contained in the microalgal cells. However, it was thought that the microbes present in the vermihumus, e.g., Clostridia, could produce lytic enzymes, including cellulases and chitinase [23-25]. These enzymes can partially digest the biomass and release nutrients for microbial growth and metabolism. CD has a low optimal concentration indicating that it does not have a key role as a substrate. Nevertheless, as CD was partly digested, it could be used as an easily assimilable substrate to shorten the lag period of the fermentation. A confirmation experiment conducted under the optimum conditions gave about 230 mL/g-VS, only around 8% lower than the predicted value (250 mL/g-VS). The volumetric methane production rate was 147.7 mL/(L·day), and the energy yield was 8.2 kJ/g-VS. The MY attained in this experiment was within the 132.3 to 411 mL/g-VS range reported in the literature (Table 6). Close inspection of the results revealed that the use of SB, *Chlorella* sp. biomass, and CD as a mixed feedstock gave higher MY than using residual sludge and sugarcane bagasse [26]. These yields were similar to that from SB and poultry manure [27]. However, with the use of readily fermentable feedstocks, e.g., food waste [28,29] and vinasse [30], methane yields were relatively higher than that observed in the present study. Curve fitting of the results showed that the lag time was relatively long (18 days), as seen in Figure 2. This was likely due to the low activity of methanogens in the vermihumus as a result of improper storage, which could lead to inactivation of the microbes [31]. The non-readily fermentable nature of substrates with no pretreatment (SCB and *Chlorella* sp. biomass) could also impede efficient hydrolysis and fermentation [6,32]. These problems might be mitigated by enriching the microbes [27] and pretreating the substrates prior to use [26]. It should, however, be noted that these methods might be energy-intensive and may add additional costs to the process.

Table 6 Methane and hydrogen production from various feedstocks reported in the literature.

Substrate	Substrate pretreatment	Fermentation process	Inoculum	Hydrogen yield	Methane yield	Reference
Sugarcane bagasse and poultry manure	No	Single stage	UASB sludge	-	229.65 NL/kg-VS	[28]
Residual sludge and sugarcane bagasse	No	Single stage	Anaerobic sludge	-	132.3 mL/g-VS	[30]
Spirulina powder and food waste	No	Single stage	Anaerobic sludge	-	390.2 mL/g-VS	[29]
Sugarcane press mud and vinasse	No	Single stage	Anaerobic sludge	-	365 L/kg-VS	[33]
Napier grass and food waste	No	Single stage	Anaerobic sludge	-	411 mL/g-VS	[34]
Sugarcane bagasse, *Chlorella* sp. biomass and cow dung	No	Single stage	Vermihumus	-	229.47 mL/g-VS	This study
Napier grass and cow dung	No	Two-stage	*Clostridium butyricum* TISTR 1032	6.98 mL/g-VS	169.87 mL/g-COD	[35]
Napier silage and cow dung	Ensiling as a pretreatment	Two-stage	*C. butyricum* TISTR 1032	27.71 mL/g-VS	141.33 mL/g-COD	[35]
Chlorella sp. biomass	Low temperature hydrothermal pretreatment (95°C, 24 h)	Two-stage	Anaerobic sludge	36.40 mL/g-VS	166.18 mL/g-VS	[36]
Chlorella sp. biomass	Hydrothermal pretreatment (3 g/L biomass, 1.5% HCl, 180°C, 15 min)	Two-stage	Anaerobic sludge	47.2 mL/g-VS	152.8 mL/g-VS	[2]
Food waste, sewage sludge and glycerol	No	Two-stage	Anaerobic sludge	140.2 mL/g-VS	342 mL/g-VS	[37]
Oil palm trunk hydrolysate	Lime pretreatment (0.2 g $Ca(OH)_2$/g-, 12°C, 60 min), followed by enzymatic hydrolysis (enzyme loading of 35 filter paper units/g)	Two-stage	*Thermoanaerobacterium thermosaccharolyticum* KKU19	136.3 mL/g-substrate	272.4 mL/g-COD	[38]
Sugarcane bagasse, *Chlorella* sp. biomass and cow dung	No	Two-stage	Vermihumus	24.41 mL/g-VS	140.76 mL/g-VS	This study

The optimal DF conditions for the two-stage process were 16.3 g-VS/L of SCB, 41.7 g-VS/L of *Chlorella* sp. biomass and 2.0 g-VS/L of CD. The predicted HY was 37.3 mL/g-VS. Again, based on Eq. (5), changing the concentration of each feedstock led to a reduction in the production of hydrogen due to the unbalanced C/N ratio of the fermentation medium. A confirmation experiment conducted under the optimum conditions gave 24.41 mL-H_2/g-VS, which was lower than the predicted yield. It is speculated that this phenomenon resulted from fluctuation of the room temperature (25°C to 37°C) during the fermentation. It is generally established that temperature can affect substrate degradation, hydrogenase activity and the metabolism of hydrogen-producing bacteria. The optimal temperatures for mesophilic DF are in a narrow range of 37°C to 40°C [39]. Therefore, decreased room temperature could possibly affect hydrogen production in an adverse manner. Additionally, the long storage time of the vermihumus might have led to changes in the activity of hydrogen-producing bacteria and the microbial community in this material, producing fluctuations in hydrogen production [40]. Further consideration of the optimal conditions revealed that the proportion of CD in the mixed feedstocks was very low (around 3%, w/w). For this reason, it is thought that CD might play an insignificant role in this biohydrogen

feedstock. An additional experiment was conducted omitting CD and the results showed that 24.28 mL-H_2/g-VS was produced. This confirmed that CD did not significantly contribute as a substrate for biohydrogen production in this experiment. It also implied that CD might play an insignificant role as the source of inoculum.

The use of the hydrogenic effluent obtained under the optimal DF conditions in the AD process yielded 140.76 mL-CH_4/g-VS. Although this was much lower than the 229.47 mL/g-VS obtained from the single-stage process, the lag time in this experiment was much shorter (6 vs. 18 days). This was possibly because volatile fatty acids (VFAs), e.g., acetate and butyrate [14], in the hydrogenic effluent could be rapidly utilized by methanogens. In the single-stage process, more time was required to hydrolyze the feedstocks and convert nutrients into VFAs for use in methanogenesis. Comparing the biohydrogen and methane production levels reported in the literature to the yields of the current experiments (Table 6), it can be seen that the mixed feedstocks used in the present study gave similar HY and MY to those obtained from Napier silage with CD [35]. However, with the use of readily fermentable substrates, e.g., food waste and glycerol [37], as well as pretreated feedstocks [35,36,38], HY and MY were much higher than the yields attained in the present study. In total, the two-stage process gave an energy yield of 5.3 kJ/g-VS, of which 0.26 kJ/g-VS was obtained from DF. Although the two-stage process gave a lower energy yield than the single-stage process, it can be used to form biohydrogen and methane that, when mixed, would yield biohythane with a hydrogen content of around 14% (v/v). Biohythane is considered superior to methane in terms of fuel performance. The presence of hydrogen in the gaseous blend increases the flame speed and fuel combustion in an engine. Biohydrogen can also diminish the carbon content in the blend, reducing the emissions of greenhouse gases resulting from fuel combustion [41].

Overall, the present study demonstrates that biohydrogen and methane can be produced using non-pretreated feedstocks. The processes used in this study omitted feedstock pretreatment, making them simpler than conventional methods. Also, by omitting pretreatment, shorter processing times and lower energy consumption are required. Coupling such advantages with the use of low-cost, readily available vermihumus as an inoculum, makes these processes more cost-effective. However, it is advisable that actual C/N ratio of the mixed feedstocks should be analyzed to determine the role of each feedstock in the process. Also, the low HY and MY observed in the current study indicates the need to improve the process performance to achieve an efficient and sustainable process for biohydrogen and methane production.

5. Conclusions

Biohydrogen and methane were successfully produced via co-digestion of SCB, *Chlorella* sp. biomass, and CD, with no substrate pretreatment. A single-stage AD process yielded 230 mL-CH_4/g-VS, equivalent to an energy yield of 8.2 kJ/g-VS. In the two-stage process, the use of mixed feedstocks in DF gave 24.41 mL/g-VS. Further use of the hydrogenic effluent for methane production yielded 140.17 mL-CH_4/g-VS. The total energy yield attained through a two-stage process was 5.3 kJ/g-VS. These results demonstrated that SCB, *Chlorella* sp. biomass and CD were feasible feedstocks for biohydrogen and methane production. However, CD had no significant effect as a substrate. Results also revealed the potential of vermihumus as an inoculum for DF and AD processes. Additionally, the present study demonstrated the applicability of a two-stage process comprised of DF and AD for biohythane production. It also reveals the possibility of attaining lower capital and operating costs for biohydrogen and methane production by omitting the pretreatment step in the process. This, coupled with the use of low-cost feedstocks (sugarcane bagasse and cow dung) and a third generation feedstock (microalgal biomass), will in turn lead to a more economical and sustainable process for biohydrogen and methane production.

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7. Conflicts of interest

The authors declare that there is no conflict of interest.

8. References

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