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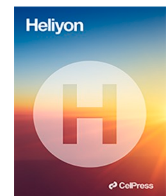

Research article

Unlocking the potential of sugarcane leaf waste for sustainable methane production: Insights from microbial pre-hydrolysis and reactor optimization

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A R T I C L E I N F O

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A B S T R A C T

Sugarcane leaf waste, a byproduct of the growing global sugar industry, challenges agricultural waste management. This study explores its potential for methane production via anaerobic digestion. A microbial pre-hydrolysis, using lignocellulose-degrading bacteria, enhanced soluble chemical oxygen demand at an optimal initial substrate concentration of 40 g-volatile solid/L. Comparative analysis with untreated and bioaugmented leaves revealed the pre-hydrolyzed leaves achieved the highest methane production rate (MPR) at 14.0 ± 0.5 mL-CH_4/L·d, surpassing others by 1.47 and 1.67 times. Two continuous stirred tank reactors were employed to assess the optimal hydraulic retention time (HRT). Results showed a stable methane production with an HRT of 25 days, yielding high MPRs: 88.70 ± 0.63 mL-CH_4/L·d from pre-hydrolyzed sugarcane leaves and 82.57 ± 1.22 mL-CH_4/L·d from microbial consortium-augmented leaves. A 25-day HRT fosters high microbial diversity with *Bacteroidota, Firmicutes, Chloroflexi*, and *Verrucomicrobiota* dominance, indicating favorable conditions. Conversely, a 20-day HRT results in lower diversity due to unfavorable factors like low pH during organic overloading, leading to increased concentrations of volatile fatty acids and lactic acid, with *Firmicutes* as the predominant phylum. This study highlights sugarcane leaf waste's potential as a valuable resource for sustainable methane production.

1. Introduction

Sugar is a globally significant food product, with over 178 million tons produced in 2019, indicating a 4.7 % growth from 2017 [1]. Thailand ranks fourth in both sugar production and exports, primarily relying on sugarcane as the raw material [2]. However, the substantial waste generated from sugarcane production, including bagasse, tops, and leaves (28 %, 17 %, and 30 % of total byproducts, respectively), poses environmental challenges. While bagasse is commonly used for power generation, the open burning of tops and leaves after harvest contributes to carbon dioxide (CO_2) emissions and global warming [3]. This underscores the urgency to develop effective technologies for managing sugarcane leaves.

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Anaerobic digestion (AD) emerges as a promising solution for treating agricultural waste, converting organic matter into valuable methane-rich biogas [4]. This complex biochemical process involves four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Hydrolysis breaks down polymeric substances into monomers, subsequently converted into volatile fatty acids (VFAs), hydrogen, and CO_2 through acidogenesis. Acetogenic bacteria transform VFAs into acetic acid, and methanogens produce methane and CO_2 in the final stage [5]. AD's potential is evident in its application to various agricultural wastes like wheat straw, corn cobs, and palm oil wastes [6–8].

Despite AD's promise, biogas production from agricultural waste faces challenges such as instability, prolonged processing time, and difficulties in degrading lignocellulosic materials, like sugarcane leaves [5,9]. To address this, pre-treatment is crucial to enhance efficiency, with physical and chemical methods being expensive and environmentally hazardous. Biological pre-treatment, using enzymes or microorganisms, is preferred for its eco-friendliness and cost-effectiveness. Studies by Ali et al. [10] and Wongfaed et al. [11] demonstrate the effective decomposition of lignocellulosic materials and enhanced methane yield (MY) through microbial consortiums.

Bioaugmentation, involving the addition of specific microorganisms to AD, has shown potential in enhancing production yields and reducing fermentation durations [12,13]. Studies by Duc et al. [12] demonstrate increased methane production under stress conditions, highlighting the capability of bioaugmentation to improve stability and reduce inhibition. Additionally, introducing microorganisms capable of producing lignocellulolytic enzymes can accelerate hydrolysis rates and increase methane production [14–17].

In the context of methane production through AD, the continuous stirred tank reactor (CSTR) is widely used [18]. Key factors influencing methane production include pH, temperature, organic loading rate (OLR), and hydraulic retention time (HRT) [19]. Maintaining an appropriate HRT is vital for optimizing methane production rate (MPR) and ensuring the stability of microbial communities in CSTR systems [20–22].

This study investigates the effects of pre-treatment on sugarcane leaves through hydrolysis, employing a microbial consortium releasing lignocellulose-degrading enzymes. It explores bioaugmentation into the AD process and evaluates methane production scaling using a CSTR with varying HRT. The outcomes aim to contribute to a selective method for incorporating the microbial consortium into the AD process and provide insights for controlling HRT in long-term biogas production from hydrolyzed sugarcane leaves and leaves augmented with the microbial consortium.

2. Materials and methods

2.1. Feedstock preparation

Sugarcane leaves were obtained from the Department of Agronomy, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. They were sun-dried until the moisture content was less than 10 % and then cut, milled, and filtered using a sieve with a mesh size of no.18 to obtain particles smaller than 1.0 mm. The resulting materials were then stored in a plastic container at room temperature (35 ± 2 °C) until they were used. The composition of sugarcane leaves is shown in Table 1.

2.2. Inoculum preparation

The cellulolytic microbial consortium (KKU-MC1) was used for the pre-hydrolysis of sugarcane leaves and bioaugmentation processes. It was isolated from rice straw compost, termite filaments, and soil samples obtained from the stalls of goats and sheep [11]. The peptone cellulose solution (PCS) medium, composed of 5 g/L peptone, 5 g/L NaCl, 2 g/L $CaCO_3$, 1 g/L yeast extract, and 3 g/L filter paper (Whatman No.1), was employed to enrich the KKU-MC1. The initial pH of the medium was adjusted to 7.5 using either 5 M HCl or 5 M NaOH. Subsequently, the medium was sterilized by autoclaving at 121 °C for 15 min. After cooling the medium to room temperature, 10 % (v/v) of KKU-MC1 was inoculated into the medium. The culture was then incubated at 37 °C and 150 rpm in an incubator shaker for 5–7 days or until the filter paper was completely decomposed. Following this, 10 % of the culture was transferred to a new PCS medium (sub-culture) and incubated under the previously described conditions. The control assay was conducted in the same manner but without adding KKU-MC1. Subsequently, the liquid samples were taken from the culture broths for enzymes activity analysis, including total cellulases, endoglucanase, exoglucanase, and hemicellulases, in which the activities were expressed in terms

Table 1
Characteristics of sugarcane leaves and inoculum.

Parameters	Sugarcane leaves	Diluted cow dung	Acclimatized cow dung
Cellulose (%)	38.89 ± 0.14	NA	NA
Hemicellulose (%)	20.13 ± 0.26	NA	NA
Lignin (%)	25.11 ± 0.05	NA	NA
Ash (%)	4.10 ± 0.09	NA	NA
Total solids (%)	94.45 ± 0.73	40.91 ± 4.01	3.03 ± 0.09
Volatile solids (%)	88.76 ± 1.56	22.07 ± 0.17	1.92 ± 0.07
Total suspended solids (%)	NA	NA	2.17 ± 0.05
Volatile suspended solids (%)	NA	NA	1.66 ± 0.04
PH	NA	NA	7.5 ± 0.0

Mean values are average of three replications ± standard deviations (n = 3).

of FPase, CMCase, Avicelase, and xylanase, respectively. The measured enzyme activity values were as follows: 0.008 ± 0.007 IU/mL, 0.029 ± 0.003 IU/mL, 0.044 ± 0.003 IU/mL, and 0.335 ± 0.098 IU/mL, respectively. The final pH of the broths was 6.03 ± 0.02.

Fresh cow dung was used to prepare an inoculum for methane production. It was diluted with distilled water at a ratio of 1:1 % (v/v) and subsequently passed through a cheesecloth to eliminate large particles. After that, 3 L of the diluted cow dung was added into a 6 L reactor. Nitrogen gas was sparged into the reactor for 20 min to create an anaerobic condition. The reactor was then incubated at 37 °C for 7 days to allow for degassing. Subsequently, the samples were taken for analysis of total solids (TS) and volatile solids (VS) (Table 1). Following the degassing process, the diluted cow dung was subjected to acclimatization before using it as an inoculum for methane production. Sugarcane leaves were employed as the substrate for this acclimatization phase. A 20 L reactor with a working volume of 10 L was carried out. Sugarcane leaves and diluted cow dung were added into the reactor at a ratio of 1:3 (VS basis). The final volume was adjusted by adding a rumen fluid solution consisting of 10 % (v/v) cow rumen in distilled water. The pH of the mixture was adjusted to 7.0 using either 5 M HCl or 5 M NaOH. Subsequently, the reactor was sealed with a rubber stopper and connected to a gas counter to measure the amount of gas generated. An anaerobic condition was created by spraying nitrogen gas into the reactor for 20 min. During acclimatization, the samples were periodically withdrawn from the reactor at 30-day intervals to measure the VS content. This VS measurement was used to calculated the amount of sugarcane leaves to be fed into the reactor, as the reactor maintained a VS ratio of 1:3 (VS basis) between the inoculum and sugarcane leaves. The acclimatization was conducted for a total of 90 days. At the end of the acclimatization period, the acclimatized inoculum was collected for analysis of pH, TS, VS, total suspended solids (TSS), and volatile suspended solids (VSS). The obtained results are shown in Table 1.

NA: Not available.

2.3. Pre-hydrolysis of sugarcane leaves using KKU-MC1

The effects of initial substrate concentration on the efficiency of pre-hydrolysis of sugarcane leaves using KKU-MC1 were investigated. The pre-hydrolysis process was performed in 120 mL serum bottles. The bottles were filled with sugarcane leaves at initial substrate concentrations of 10, 20, 30, 40, and 50 g-VS/L. After that, 50 mL of sterile PCS medium excluded filter paper was added to each bottle. The bottles were tightly sealed with rubber stoppers and aluminum caps. Aseptically, a 10 % (v/v) solution of KKU-MC1 was injected into each bottle. The bottles were then incubated at 37 °C and 150 rpm for 5 days [11,23]. The control test followed the same procedure, but without the addition of KKU-MC1. The experiment was performed with 4 replicates. Following the pre-hydrolysis process, the collected samples comprising both liquid and solid fractions were designated as sugarcane leaf hydrolysate, which were subsequently employed as substrates for methane production. Additionally, this hydrolysate underwent characterization analysis, wherein the solid fraction was utilized for VS analysis, while the liquid hydrolysate was examined for pH, total chemical oxygen demand (tCOD), soluble chemical oxygen demand (sCOD), enzyme activity (cellulase, endoglucanase, exoglucanase, and xylanase), reducing sugar, and VFAs.

2.4. Methane production from sugarcane leaves in batch fermentation

Batch fermentation experiments were conducted to evaluate methane production from sugarcane leaves pre-hydrolyzed with KKU-MC1, and from sugarcane leaves augmented with KKU-MC1. The sugarcane leaf hydrolysate obtained from the optimal substrate concentration of the pre-hydrolysis step was used to investigate the impact of pre-hydrolysis. Concurrently, the sugarcane leaves and KKU-MC1 were used to test the augmentation effect under the optimal substrate concentration from pre-hydrolysis. The fermentation process was conducted in a 120 mL serum bottle with a working volume of 70 mL. Each bottle was filled with the methanogenic inoculum at a ratio of 1:1 (VS basis). The pH of the mixture was adjusted to 7.0 using 1 M HCl or 1 M NaOH. The bottle was tightly sealed with a rubber stopper and an aluminum cap, then flushed with nitrogen gas for 10 min to remove oxygen from the headspace. Subsequently, it was incubated in an orbital incubator shaker at 37 °C and 150 rpm for 45 days. A treatment containing sugarcane leaves and methanogenic inoculum was used as a control to determine the efficiency of methanogenic inoculum on the untreated material. The treatment containing only methanogenic inoculum was used to determine biogas production from inoculum. Additionally, a treatment control to evaluate biogas production from KKU-MC1 was also implemented. This control involved each sugarcane leaf hydrolysate and sugarcane leaves augmented with KKU-MC1 but without methanogenic inoculum. All treatments were performed in quadruplicates. During fermentation, the volume of biogas was measured daily using a 50 mL glass syringe, and the composition of biogas was evaluated using gas chromatography (GC).

2.5. Simulation of the semi-continuous process by the kinetic model

The cumulative MY obtained from batch fermentation was used to predict the HRT in semi-continuous fermentation, following the method previously described by Caillet and Adelard [24]. The cumulative MY (A, mL-CH_4/g-VS) at a certain fermentation time (t, days) during the exponential phases, and the maximum MY (A_{max}, mL-CH_4/g-VS) were used to calculate the rate constant of first-order kinetic (k, days^{-1}) using Eq. (1). Following that, the calculated k and the defined absolute proportion (p) at 80 % of the maximal MY (A_{max}, mL-CH_4/g-VS) were used to estimate the HRT (days) according to Eq. (2) [25].

$$A(t) = A_{max} + \left(1 - e^{-kt}\right) \tag{1}$$

$$\mathrm{HRT} = \frac{\rho}{k(1-\rho)} \tag{2}$$

2.6. Methane production from sugarcane leaves in semi-continuous fermentation

Methane production was scaled-up using a 2 L CSTR with a working volume of 1.5 L (Fig. 1a). Two reactors, namely R1 and R2, were set up and operated to compare the efficiency of methane production between pre-hydrolyzed sugarcane leaves and sugarcane leaves augmented with KKU-MC1. In the start-up phases, R1 was fed with sugarcane leaf hydrolysate obtained from the pre-hydrolysis process, whereas R2 was fed with a mixture of sugarcane leaves and KKU-MC1. The methanogenic inoculum was introduced into both reactors at a ratio of 1:1 (VS basis). The initial pH of the mixture was adjusted to 7.0 using either 1 M HCl or 1 M NaOH. The reactors were tightly sealed and then purged with nitrogen gas for 10 min to achieve anaerobic conditions. The reactors were started up in batch fermentation mode for 20 and 30 days for R1 and R2, respectively, at 37 ± 5 °C and 150 rpm. Subsequently, the reactors were switched to semi-continuous fermentation mode, with sugarcane leaf hydrolysate being fed to R1 and raw sugarcane leaves for R2. This process was conducted while maintaining the HRT of 40 days. Thereafter, HRT was stepwise reduced to 30, 25, and 20 days for R1 and gradually decreased to 30 and 20 days for R2. In each HRT, the fermentation was conducted until the biogas production rate did not vary by more than 10 % from its average value [26]. During semi-continuous fermentation, the pH of the fermentation broth was controlled at 7.0 ± 0.2 by adding 1 M NaOH or 1 M HCl. The biogas volume was recorded and its composition was analyzed daily using GC. Meanwhile, the concentrations of VFAs and alkalinity were measured every two days. The CSTR system setup is shown in Fig. 1b.

2.7. Microbial community analysis

The sludge samples from the bioaugmentation reactor under the optimal HRT of 25 days and an organic overloading of 20-day HRT were withdrawn and stored at −20 °C prior to DNA extraction. Genomic DNA was extracted using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA, USA), and its concentration and purity were determined using UV–Vis Spectrophotometer NanoDrop 300 (Nano-300, Hangzhou Allsheng Instruments) at 260 (A260) and 280 (A280) nm wavelengths, with acceptability for purity defined as 1.8–2.0 (A260/A280). The extracted DNA was then amplified using the polymerase chain reaction (PCR) with the universal primer pair: 515F (5′-GTG CCA GCM GCC GCG GTA A-3′) and 806R (5′-GGA CTA CNN GGG TAT CTA AT-3′), targeting the conserved bacterial 16S rRNA gene. The quality of PCR products was assessed using the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Consequently, the 16S rRNA libraries were sequenced on the IlluminaHiSeq platform 2500 by Novogene (Beijing, China). The sequencing process analysis involved reads being clustered and dereplicated into amplicon sequence variables (ASVs) using the q2-dada2 plugin and the denoise-single method within the QIIME2 pipeline version 2020.2 [27]. Taxonomy assignment of the ASVs was performed using QIIME2 against the SILVA database version 138.1 [28]. Functional predictions abundance was performed using PICRUSt2 version 2.3.0 [29].

2.8. Analytical methods

The contents of TS, VS, TSS, VSS, tCOD, sCOD, and ash were analyzed in accordance with standard methods for water and wastewater examination [30]. The sample for tCOD consisted of the hydrolysate withdrawn from the reaction using a 1 mL syringe, which contained organic components in both liquid and solid fractions. Meanwhile, the samples for sCOD were prepared by centrifuging the hydrolysate at 10,000 rpm for 5 min and then filtering the supernatant through a 0.45 μm cellulose acetate membrane filter. The contents of cellulose, hemicellulose, and lignin were determined using the method outlined by Goering and Van Soest [31]. The pH

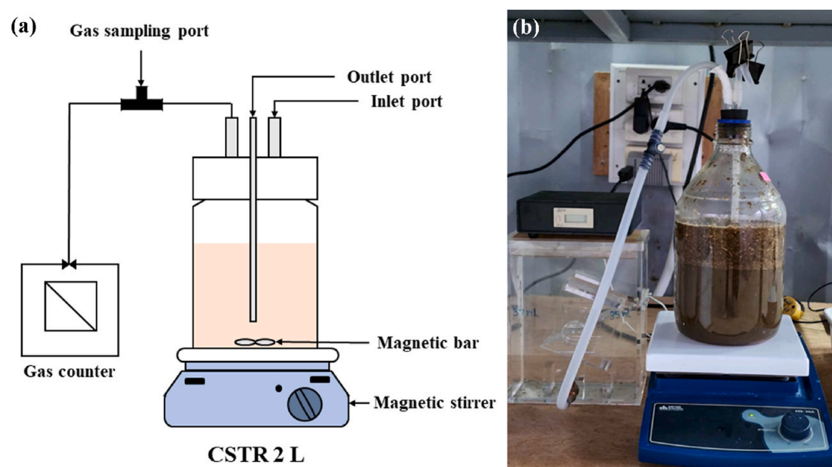

Fig. 1. System for methane production using a continuous stirred tank reactor (CSTR); (a) schematic diagram and (b) image.

Table 2
Parameters of sugarcane leaf hydrolysate after pre-hydrolysis using KKU-MC1 with various initial substrate concentrations of 10, 20, 30, 40, and 50 g-VS/L.

Sample (g-VS/L)	VS removal (%)	Enzyme activity (IU/ml)				tCOD (g/L)	sCOD (g/L)	Reducing sugar (g/L)	VFAs concentration (g/L)				pH	
		FPase	CMCase	Avicelase	Xylanase				Acetic acid	Propionic acid	Butyric acid	Total VFAs	Initial	Final
10	2.42 ± 0.61^{d}	0.008 ± 0.003^{ab}	0.022 ± 0.002^{c}	0.033 ± 0.004^{c}	0.274 ± 0.001^{a}	10.29 ± 0.10^{d}	8.90 ± 0.08^{d}	0.26 ± 0.00^{e}	1.51 ± 0.33^{d}	0.53 ± 0.11^{c}	0.34 ± 0.13^{c}	2.38 ± 0.45^{e}	7.30 ± 0.10^{ab}	6.57 ± 0.05^{ab}
20	7.76 ± 0.64^{c}	0.007 ± 0.004^{b}	0.028 ± 0.003^{b}	0.032 ± 0.003^{c}	0.275 ± 0.001^{a}	11.28 ± 0.15^{c}	10.24 ± 0.13^{c}	0.66 ± 0.00^{d}	2.27 ± 0.00^{a}	0.46 ± 0.00^{d}	0.33 ± 0.00^{d}	3.06 ± 0.45^{c}	7.27 ± 0.06^{c}	6.33 ± 0.05^{ab}
30	13.19 ± 0.94^{b}	0.008 ± 0.002^{a}	0.012 ± 0.003^{d}	0.032 ± 0.003^{c}	0.284 ± 0.012^{a}	11.59 ± 0.15^{b}	10.65 ± 0.05^{b}	0.70 ± 0.01^{c}	1.87 ± 0.12^{c}	0.69 ± 0.02^{b}	0.33 ± 0.01^{d}	2.89 ± 0.80^{d}	7.37 ± 0.35^{a}	6.47 ± 0.05^{ab}
40	18.92 ± 2.12^{a}	0.008 ± 0.007^{a}	0.029 ± 0.003^{a}	0.039 ± 0.003^{b}	0.287 ± 0.098^{a}	12.16 ± 0.04^{a}	11.24 ± 0.08^{a}	0.78 ± 0.00^{a}	1.87 ± 0.46^{c}	0.97 ± 0.15^{a}	1.17 ± 0.23^{a}	4.01 ± 0.39^{a}	7.20 ± 0.10^{bc}	6.20 ± 0.36^{b}
50	18.93 ± 0.72^{a}	0.003 ± 0.006^{c}	0.008 ± 0.005^{e}	0.042 ± 0.002^{a}	0.265 ± 0.003^{a}	8.80 ± 0.00^{e}	7.98 ± 0.07^{e}	0.76 ± 0.00^{b}	1.94 ± 0.06^{b}	0.69 ± 0.02^{b}	0.97 ± 0.02^{b}	3.61 ± 0.84^{b}	7.23 ± 0.15^{ab}	6.50 ± 0.20^{a}

Mean values are average of four replications ± standard deviations ($n = 4$).
Values marked with the same alphabets are not significantly different ($p < 0.05$).

was determined with a pH meter (pH-500, Queen, USA). The activities of FPase, CMase, avicelase, and xylanase were analyzed based on the procedures outlined by the IUPAC Commission of Biotechnology [32]. The concentrations of acetic acid, propionic acid, butyric acid, and lactic acid, were analyzed by high-performance liquid chromatography (HPLC) (Shimadzu LC- 20AD, Kyoto, Japan) with a 7.80 × 300 mm Vertisep™ OA column (Vertical Chromatography, Thailand) and a refractive index (RI) detector [33]. The total VFAs and alkalinity were measured according to the standard methods [30]. The volume and composition of the biogas were measured using a 50 mL glass syringe and GC (Shimadzu GC-2014, Kyoto, Japan) with a thermal conductivity detector (TCD), respectively. The operating conditions of the GC were as described in Sitthikitpanya et al. [34]. The MY was determined by dividing the cumulative volume of methane produced (mL CH_4) by the total volume of substrate added (VS_{added}). The MPR was calculated by dividing the volume of methane produced (mL CH_4) by the working volume (L) and fermentation time (days) [35]. The lag time (λ) (days) was determined using the modified Gompertz model [36].

Statistical analysis was performed using IBM SPSS Statistics 25 software (SPSS, Inc., Chicago, IL, USA). An analysis of variance (ANOVA) test followed by Duncan's multiple range test was used at a significance level of $p < 0.05$.

3. Results and discussion

3.1. Impact of substrate concentration on pre-hydrolysis

Table 2 presents the characteristics of sugarcane leaf hydrolysate obtained after pre-hydrolysis with KKU-MC1 at different initial substrate concentrations. The reduction in VS content of the pre-hydrolyzed sugarcane leaves ranged from 2.42 ± 0.61 % to 18.93 ± 0.72 %. An increase in substrate concentration resulted in a gradual increase in VS reduction. The highest VS reduction of 18.93 ± 0.72 % was achieved with the maximum substrate concentration of 50 g-VS/L. However, this value was not significantly different ($p > 0.05$) from the VS reduction (18.92 ± 2.12 %) obtained with a substrate concentration of 40 g-VS/L. The enhanced VS reduction at higher substrate concentration may be attributed to the increased organic availability, which could be hydrolyzed by microbes. Nevertheless, higher substrate concentration also led to elevated solid content and viscosity of the medium [37]. These factors negatively influenced the mixing between microbes and the substrate, consequently affecting microbial activities. An analysis of enzyme activities indicated that the highest FPase was 0.008 ± 0.007 IU/mL, CMCase was 0.029 ± 0.003 IU/mL, and xylanase was 0.287 ± 0.098 IU/mL, all observed at an initial substrate concentration of 40 g-VS/L. Conversely, an increase in substrate concentration to 50 g-VS/L resulted in decreased FPase, CMCase and xylanase, with only a slight increase in avicelase.

Subsequently, the poor mixing and low enzymes release from microbes led to a limited solubilization of organic matter from the sugarcane leaves into the liquid hydrolysates. It was observed that the tCOD, sCOD, total VFAs and reducing sugar reached the maximal values at 12.16 ± 0.04 g/L, 11.24 ± 0.08 g/L, 4.01 ± 0.39 g/L, and 0.78 ± 0.00 g/L, respectively, when the substrate concentration was performed at 40 g-VS/L. However, with a gradual increase in substrate concentration to 50 g-VS/L, the obtained results of tCOD, sCOD, total VFAs and reducing sugar were significantly lower ($p < 0.05$) compared to those obtained at a substrate concentration of 40 g-VS/L. The concentration of total VFAs correlated with the final pH, as the final pH of the hydrolysates from the 40 g-VS/L substrate concentration reached the lowest value of 6.20 ± 0.36. Based on the results obtained, the pre-hydrolysis process using KKU-MC1 at an initial substrate concentration of 40 g-VS/L demonstrated the highest efficiency in the biodegradation of sugarcane leaves. This condition led to the greatest solubilization of organic matters, as evidenced by higher values of tCOD, sCOD, total VFAs and reducing sugar. In contrast, a higher substrate concentration of 50 g-VS/L exhibited inadequate mass transfer, resulting in lower biodegradability efficiency, and consequently lower release of hydrolyzed products during the pre-hydrolysis process. The results are consistent with the research by Hebbale and Ramachandra [38], who reported that high substrate concentrations lead to a decrease in the concentration of reducing sugars. In their study on the acid hydrolysis process of macroalgae, they found that as the substrate concentration increased from 10 to 50 g/L, the concentration of reducing sugars also increased. However, when the substrate concentration exceeded 50 g/L, the concentration of reducing sugars decreased significantly. Wang et al. [39] also reported that both sCOD and VFAs were increased as substrate concentrations rose up to 200 g/L and decreased when concentrations exceeded this level. The decline in sCOD and VFAs at higher concentrations could be attributed to factors such as difficult stirring and inadequate mass transfer.

The composition of individual VFAs during the biodegradation process is influenced by various factors such as substrate concentration, substrate characteristics, pH, inoculum type, and the expression of functional enzymes in the microbial community [40]. In our study, the substrate concentration played a significant role in affecting the quantities of individual VFAs. As depicted in Table 2, acetic acid was the predominant VFA across all substrate concentrations, ranging from 1.51 ± 0.33 g/L to 2.27 ± 0.00 g/L. This was followed by propionic acid, ranging from 0.46 ± 0.00 g/L to 0.97 ± 0.15 g/L, and butyric acid, ranging from 0.33 ± 0.00 g/L to 1.17 ± 0.23 g/L. The highest concentration of acetic acid (2.27 ± 0.00 g/L) was observed at a substrate concentration of 20 g-VS/L, with a slight decrease as the substrate concentration increased to 30–50 g-VS/L. Regarding propionic acid and butyric acid, their maximal concentrations of 0.97 ± 0.15 g/L and 1.17 ± 0.23 g/L, respectively, were detected at a substrate concentration of 40 g-VS/L. This demonstrates that increasing the substrate concentration results in a change in VFAs composition. These findings align with the research of Wang et al. [39], which reported that differing substrate concentrations distinctly influence both the quantity and composition of VFAs generated. On the other hand, Pu et al. [41] found that acetic acid increases as the substrate concentration rises. However, when the substrate concentration exceeds 7.5 g-VS/L, the acetic acid noticeably decreases while the butyric acid increases.

3.2. Methane production from sugarcane leaves in batch fermentation

The cumulative MY and kinetic parameters of methane production from sugarcane leaf hydrolysate, sugarcane leaves augmented with KKU-MC1, and raw sugarcane leaves are shown in Fig. 2 and Table 3. It can be observed that there was no detectable methane production in the conditions of sugarcane leaf hydrolysate and sugarcane leaves augmented with KKU-MC1 without the addition of acclimatized cow dung (Fig. 2), indicating the absence of methanogens in the KKU-MC1.

Regarding the methane production from sugarcane leaf hydrolysate, sugarcane leaves augmented with KKU-MC1, and raw sugarcane leaves, inoculated with acclimatized cow dung (Fig. 2), it was observed that methane production exhibited a rapid increase from day 1 to day 20 of fermentation. The modified Gompertz model indicated the absence of a lag phase ($\lambda = 0$ days) for all three conditions (Table 3). These findings suggested that the acclimatized cow dung quickly adapted to digest the organic matter present in both soluble forms (such as sCOD, VFAs and reducing sugar in the hydrolyzed sugarcane leaves) and insoluble forms (cellulose and hemicellulose in sugarcane leaves). These results might suggest that the fast conversion process in digesting substrates to producing methane of microbial communities in the acclimatized cow dung might benefit from the acclimatization step. Similar results were observed by Kurade et al. [42], who reported that methane production from acclimatized sludge in the initial phase of AD yielded higher methane content (65–76 %) than unacclimatized sludge (26–73 %). Additionally, rumen fluid was added into the acclimatized cow dung during the acclimatization process. It has been previously reported that rumen bacteria secrete cellulolytic enzymes that facilitate the hydrolysis and acidogenesis of lignocellulosic materials. For instance, research by Liang et al. [43] found that rumen fluid can efficiently degrade lignocellulosic biomass, with microorganisms in the rumen playing a crucial role. This research also revealed that the digestion of rice straw with added rumen fluid resulted in the efficient degradation of cellulose, hemicellulose, and lignin, reaching up to 46.2 %, 60.4 %, and 12.9 %, respectively. Moreover, a report by Meyer et al. [44] showed that the addition of rumen fluid into the AD of barley straw could decrease the digestion time while increasing the substrate degradation rate.

The MPR ranging from 8.4 ± 0.1 mL-CH_4/L·d to 14.0 ± 0.5 mL-CH_4/L·d was achieved. The highest MPR of 14.0 ± 0.5 mL-CH_4/L·d was observed when sugarcane leaf hydrolysate was used as a substrate. This value was 1.47 and 1.67 times higher than the MPR obtained from the digestion of sugarcane leaves augmented with KKU-MC1 and raw sugarcane leaves, respectively. This difference can be attributed to the higher concentration of readily degradable organic matter, including sCOD, reducing sugar and VFAs, present in the hydrolysate (Table 2), which facilitated a faster and more efficient conversion to methane. In contrast, the lower MPR observed for sugarcane leaves augmented with KKU-MC1 and raw sugarcane leaves can be attributed to the slower digestion of the solid fraction. Typically, the digestion of solid fractions begins after the digestion of readily soluble organic matter [45]. Similar results were reported by Ref. [46], who found that pre-hydrolysis of extruded waste wood with microbes before the AD step resulted in a 2.28-fold increase in the MPR. Another study by Ref. [47] reported that microbial pre-hydrolysis affected the digestion of complex lignocellulosic substrates by increasing the population of bacteria capable of hydrolysis and acidogenesis, leading to an enhanced MPR.

The methane production process reached completion on day 45 (Fig. 2). The highest MY of 199.3 ± 7.0 mL-CH_4/g-VS_{added} was obtained from sugarcane leaf hydrolysate. However, this value did not exhibit a significant difference ($p > 0.05$) compared to the MY of 195.9 ± 2.5 mL-CH_4/g-VS_{added} obtained from the sugarcane leaves augmented with KKU-MC1. On the other hand, the lowest MY of 190.6 ± 3.7 mL-CH_4/g-VS_{added} obtained from raw sugarcane leaves was significantly lower ($p < 0.05$) than those obtained from sugarcane leaf hydrolysate and sugarcane leaves augmented with KKU-MC1. These findings highlight that the pre-hydrolysis and bioaugmentation of sugarcane leaves using KKU-MC1 can significantly enhance MY compared to the condition without the addition of KKU-MC1. This can be attributed to the presence of various bacteria within KKU-MC1, including *Parabacteroides*, *Bacteroides*,

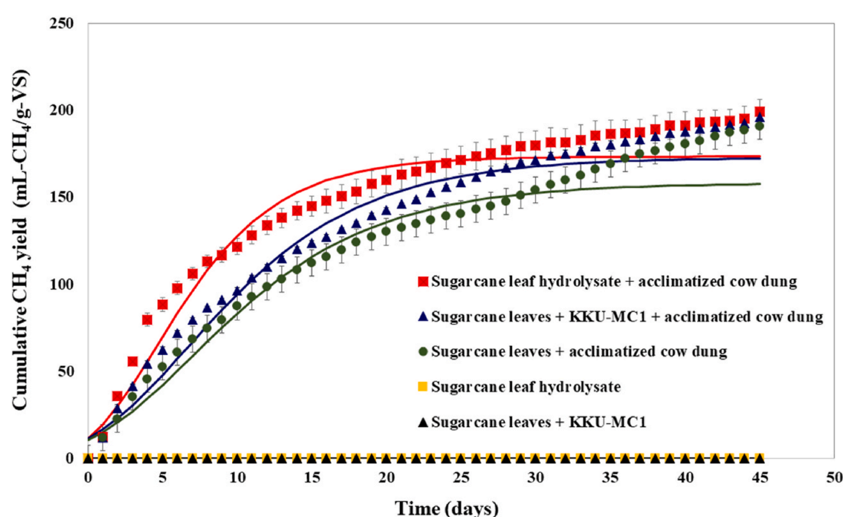

Fig. 2. Cumulative methane yield from batch fermentation of sugarcane leaf hydrolysate, sugarcane leaves augmented with KKU-MC1, and raw sugarcane leaves.

Table 3
Kinetic parameters of methane production from batch fermentation.

Conditions	MY ($mL-CH_4/g-VS_{added}$)	MPR (mL/L·d)	λ (d)	R^2
Sugarcane leaf hydrolysate + acclimatized cow dung	199.3 ± 7.0^{a}	14.0 ± 0.5^{a}	0.0 ± 0.0^{a}	0.961
Sugarcane leaves + KKU-MC1 + acclimatized cow dung	195.9 ± 2.5^{ab}	9.5 ± 0.2^{b}	0.0 ± 0.0^{a}	0.978
Sugarcane leaves + acclimatized cow dung	190.6 ± 3.7^{b}	8.4 ± 0.1^{c}	0.0 ± 0.0^{a}	0.981

MY: Methane yield, MPR: Methane production rate, λ: Lag-phase time, R^2: Coefficient of determination.

Pseudomonas, and *Ruminiclostridium* [11], which play essential roles in the hydrolysis and acidogenesis of lignocellulosic conversion.

The comparison of the effects of pre-hydrolysis and bioaugmentation using KKU-MC1 on methane production clearly showed that pre-hydrolyzing sugarcane leaves with KKU-MC1 prior to their use as a substrate significantly enhances the efficacy of methane production, as evidenced by the higher MPR and MY (Table 3). This was because, during the pre-hydrolysis process, microbes released lignocellulolytic enzymes such as cellulases, xylanases, and β-glucosidases. These enzymes functioned to break down the large polymer molecules of the substrate into smaller molecules such as oligomers and monomers, leading to the release of dissolved compounds that were readily accessible to methanogenic bacteria during the AD process [11]. As a result, there was increased in the MPR and MY.

Although the MY from bioaugmentation did not differ significantly from that of pre-hydrolysis, the MPR was significantly lower. MPR is a critical parameter for evaluating biodigestibility efficiency during the AD process, providing insights into the extent of hydrolysis [48]. In our study, the lower MPR in the bioaugmentation process may be attributed to an inappropriate dose of KKU-MC1 loaded into the AD process. A study by Linsong et al. [49] demonstrated that increasing bioaugmentation seed (BS) doses from 0.07 $g-VS_{BS}/g-VS_{substrate}$ to 0.14, 0.21, 0.27, and 0.34 $g-VS_{BS}/g-VS_{substrate}$ resulted in higher methane production. However, their study revealed that adding excessive amounts of BS at the highest dose of 0.34 $g-VS_{BS}/g-VS_{substrate}$ did not significantly improve efficiency compared to the lower dose of 0.27 $g-VS_{BS}/g-VS_{substrate}$. Therefore, our current study suggests that further research should focus on determining the appropriate loading quantities of KKU-MC1 as bioaugmentation seed cultures. Additionally, it is important to note that even though the bioaugmentation process resulted in a significantly lower MPR compared to pre-hydrolysis, the overall processing time for bioaugmentation (45 days) was shorter than that for pre-hydrolysis combined with methane production (50 days). Therefore, to identify the most appropriate conditions for methane production from sugarcane leaves, a techno-economic analysis is suggested to assess the input and output materials associated with both processes. Furthermore, a long-term fermentation process could be conducted, as the microbes in the bioaugmentation reactor may adapt and create stable microbial communities over prolonged periods.

Comparing the MY obtained from this study with the literature, where sugarcane leaves were used as the substrate (Table 4). The MY obtained from this study (199.3 $mL-CH_4/g-VS$) are comparable to the yields from other studies, ranging from 81.0 $mL-CH_4/g-VS$ to 223.6 $mL-CH_4/g-VS$. However, the yield obtained from our research is relatively low compared to that of Sitthikitpanya et al. [50] (346.7 $mL-CH_4/g-VS$), which could be due to differences in pre-treatment methods and fermentation processes. MY can be influenced by several factors including the type of substrate used, the pre-treatment methods employed, fermentation processes, loading rates of bacteria and organic material, as well as the specific microbial consortium present. Therefore, the selection of appropriate substrate pre-treatment methods and the proper control of other factors are critical to enhance the efficiency of methane production.

3.3. Methane production from sugarcane leaves in semi-continuous fermentation

The cumulative MY obtained from batch experiments using sugarcane leaf hydrolysate and sugarcane leaves augmented with KKU-MC1 were employed to estimate the k, resulting in values of 0.2024 $days^{-1}$ and 0.1584 $days^{-1}$, respectively. Subsequently, these k values were used to estimate the HRT for semi-continuous methane production, resulting in an estimated HRT of 20 days for sugarcane leaf hydrolysate and 25 days for sugarcane leaves augmented with KKU-MC1.

Fig. 3 presents time-course profiles of MPR, methane content, VFA/alkalinity ratio, pH and concentrations of VFAs and lactic acid

Table 4
Comparing the methane yield (MY) from this study with relevant literature reviews where sugarcane leaves were used as the substrate.

Pre-treatment method	Fermentation mode	MY ($mL-CH_4/g-VS$)	References
Alkaline pretreatment (2 % (w/v) NaOH, 60 min) + enzymatic hydrolysis (cellulase 15 FPU/g-sugarcane leaves, 72 h)	Two-stage	346.7	[50]
Deep eutectic solvent pre-treatment (120 °C, 3 h)	Two-stage	118.0	[23]
	One-stage	81.0	
Alkaline pre-treatment (10 % (w/v) NaOH, 120 min) + fungal pre-treatment (*Xylaria* sp. BCC 1067, 28 days)	One-stage	165.5	[9]
Liquid hot water pre-treatment (170 °C, 15 min)	One-stage	199.8	[51]
Dilute acid pretreatment (2.5 % (w/w) H_2SO_4, 150 °C, 15 min)	One-stage	205.3	
Potassium hydroxide pretreatment (10 % (w/w) KOH, 150 °C, 60 min)	One-stage	223.6	
No pre-treatment	One-stage	165.4	
No pre-treatment	One-stage	164.4	[52]
Biological pre-hydrolysis (10 % (v/v) KKU-MC1, 5 days)	One-stage	199.3	This study
No pre-treatment	One-stage	190.6	

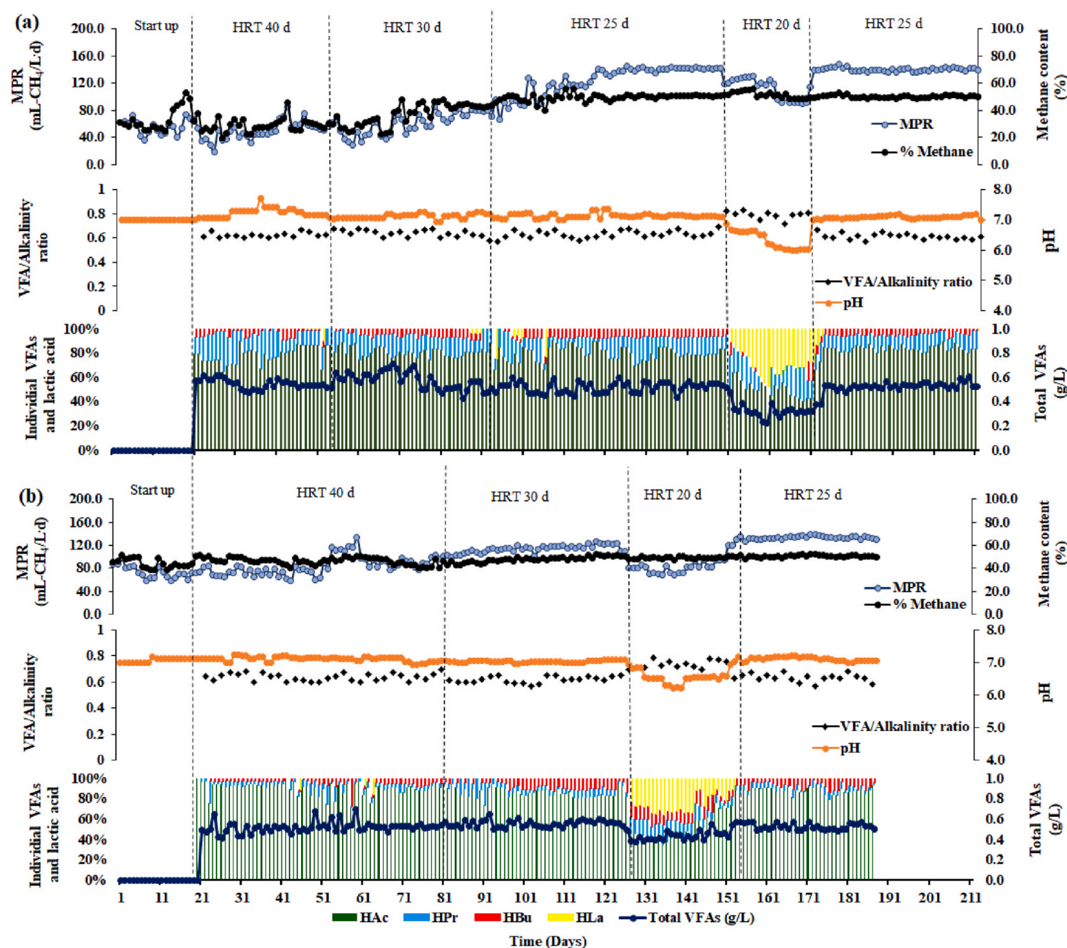

Fig. 3. Time-course profiles of methane production rate (MPR), methane content, volatile fatty acid (VFA)/alkalinity ratio, pH and concentrations of VFAs and lactic acid during the semi-continuous fermentation of (a) sugarcane leaves pre-hydrolyzed with KKU-MC1 and (b) sugarcane leaves augmented with KKU-MC1.

during the semi-continuous fermentation of sugarcane leaves pre-hydrolyzed with KKU-MC1 (Fig. 3a) and sugarcane leaves augmented with KKU-MC1 (Fig. 3b). During the start-up phase of the semi-continuous fermentation of sugarcane leaves pre-hydrolyzed with KKU-MC1 (Fig. 3a), methane content ranging from 25 % to 33 % was detected from day 1 to day 12.

Table 5
Parameters of methane production from sugarcane leaves pre-hydrolyzed with KKU-MC1 in each HRT under steady-state conditions.

Parameters	HRT (days)				
	40	30	25	20	25[a]
Biogas production rate (mL/L·d)	158.67 ± 14.76	171.82 ± 18.88	280.00 ± 16.04	186.67 ± 16.50	280.00 ± 14.07
MPR (mL-CH_4/L·d)	43.24 ± 6.21	74.59 ± 6.77	141.92 ± 1.00	92.28 ± 2.85	141.74 ± 3.38
Biogas yield (mL/g-VS)	156.58 ± 14.56	128.86 ± 14.16	175.00 ± 5.90	93.33 ± 8.25	175.00 ± 8.79
MY (mL-CH_4/g-VS)	52.03 ± 9.50	39.87 ± 9.46	88.70 ± 0.63	68.12 ± 5.05	88.59 ± 2.11
Methane content (%)	27.37 ± 4.06	43.54 ± 1.87	50.69 ± 0.36	49.44 ± 1.52	50.62 ± 1.21
pH	7.33 ± 0.16	7.17 ± 0.08	7.12 ± 0.02	6.09 ± 0.17	7.07 ± 0.02
VFA (g/L)	6.30 ± 0.17	5.80 ± 0.52	5.50 ± 0.09	6.60 ± 0.09	5.10 ± 0.52
Alkalinity (g/L)	10.00 ± 0.35	9.10 ± 0.17	8.70 ± 0.23	8.40 ± 0.17	8.40 ± 0.52
VFA/Alkalinity ratio	0.63	0.64	0.63	0.79	0.61
Acetic acid (g/L)	0.41 ± 0.03	0.41 ± 0.05	0.41 ± 0.03	0.22 ± 0.03	0.42 ± 0.05
Propionic acid (g/L)	0.11 ± 0.06	0.07 ± 0.02	0.08 ± 0.01	0.09 ± 0.03	0.06 ± 0.01
Butyric acid (g/L)	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.02 ± 0.03	0.03 ± 0.02
Lactic acid (g/L)	0.00 ± 0.00	0.01 ± 0.01	0.03 ± 0.02	0.18 ± 0.02	0.02 ± 0.01

MPR: Methane production rate, MY: Methane yield, VFA: Volatile fatty acid.
[a] Confirmation of the optimal HRT results.

Subsequently, there was a gradual rise to 52 % by day 17. This increase was corresponded to an increase in MPR, reaching 73 mL-CH_4/L·d. These observations suggests that during day 1 to day 12, the microbes were acclimatized and adapted themselves to the substrate and new environments. Following reactor start-up, the HRT of 40 days was adjusted at day 21 and then stepwise decreased to 30, 25 and 20 days (Fig. 3a). The decrease in HRT from 40 to 30 and 25 days led to a gradual continuous rise in MPR (Table 5), from 43.24 ± 6.21 to 74.59 ± 6.77 and 141.92 ± 1.00 mL-CH_4/L·d, respectively, at the steady state. Similar trends were observed in term of methane content, wherein the methane contents increased from 27.37 ± 4.06 to 43.54 ± 1.87 and 50.69 ± 0.36 % with the reduction of HRT from 40 to 30 and 25 days, respectively. However, as the HRT was further decreased to 20 days, the MPR was dropped to 92.28 ± 2.85 mL-CH_4/L·d. These declines in MPR were correlated with a decrease in pH (6.09 ± 0.17) caused by accumulation of VFAs (6.60 ± 0.09 g/L). The pH reduction and accumulation of VFAs indicate organic overloading within the system. Under conditions of high organic loading, both hydrolytic bacteria and acidogenic bacteria are generally growth and active, leading to an enhanced hydrolysis and acidogenesis of the organic substrate. An elevated levels of accumulated VFAs and a resulting low pH can suppress methanogens activity, thereby causing process failure [53]. The detected pH (6.09 ± 0.17) of the HRT of 20 days was lower than the suitable pH range of 6.5 − 7.5 recommended for maintaining a stable methane fermentation process [26]. Furthermore, the VFA/alkalinity ratio at the HRT of 20 days was relatively high, reaching 0.79. This VFA/alkalinity ratio could serve as an indicator of the buffering capacity of the system, as the increase in VFAs and a decline in alkalinity signifies a diminished pH buffer capacity. Generally, a maximum threshold VFA/alkalinity ratio of 0.6 is considered suitable to maintain a healthy AD process without the risk of acidification [19]. Therefore, the high VFA/alkalinity ratio could potentially emerge as a critical factor contributing to the instability observed in the 20-days HRT. In contrast, it was observed that the VFA/alkalinity ratio, between 0.63 and 0.64, detected within the HRTs of 40, 30 and 25 days, stayed within a range that closely aligns with favorable conditions (0.6) [19]. This observation is consistent with previous research that indicated a VFA/alkalinity ratio of 0.6, which was detected at the optimal HRT and resulted in effective biogas production from biogas effluent and filter cake [54].

The total concentration of acetic, butyric, and propionic acids in the effluent at each HRT ranged from 0.43 to 0.53 g/L (Fig. 3a), which falls within the appropriate range for a stable methanogenic fermentation process, <1 g/L, as reported by Guneratnam et al. [55]. Acetic acid was the primary metabolite found in effluent, with concentrations ranging from 0.22 to 0.45 g/L (Table 5). Additionally, propionic acid (0.06–0.11 g/L) and butyric acid (0.02–0.03 g/L) were also detected at low concentrations. Typically, methanogenic bacteria preferentially utilize acetic acid for methane production more than propionic and butyric acids. Therefore, the absence of accumulation of propionic and butyric acids in the system indicates that the methanogenesis process is operating effectively. However, at the HRT of 20 days, lactic acid was observed (0.06–0.21 g/L) (Fig. 3a). The accumulation of lactic acid in the effluent can lead to toxicity to methanogens and inhibition of methanogenic activity, resulting in decreased methane production efficiency [56,57]. Additionally, high lactic acid concentrations may indicate process instability or unfavorable conditions for methane-producing microorganisms, thereby impeding the overall performance of methane production. Cheng et al. [57] investigated approaches for enhancing the degradation of lactic acid to mitigate methane production inhibition in AD. They discovered that the use of nanoscale zero-valent iron can enhance the degradation of lactic acid for methane production. Furthermore, short HRTs may lead to a washout of microorganisms, resulting in decreased methane production. Bi et al. [58] investigated the impact of HRT on anaerobic co-digestion of food waste and cattle manure. They found that at a low HRT of 20 days, the fermentation process failed extensively due to the accumulation of VFAs and the washout of microorganisms, resulting in reduced methane production.

Fig. 3b illustrates the time-course profiles of methane production from sugarcane leaves augmented with KKU-MC1 through semi-continuous fermentation. The reduction of HRT from 40 to 30 days resulted in a noticeable increase in MPR, rising from 74.86 ± 6.80 to 120.86 ± 3.86 mL-CH_4/L·d (Table 6). Subsequently, when HRT was further decreased to 20 days, the MPR decreased significantly to 72.18 ± 4.88 mL-CH_4/L·d. However, upon extending the HRT to 25 days, the MPR increased to 135.43 ± 2.15 mL-CH_4/L·d, surpassing the MPR obtained from the 30-days HRT. These results are consistent with the methane content, revealing that at a 25-days HRT, methane production reached its maximum content of 51.30 ± 0.64 %. The pH values at steady-state conditions of the effluent at 40-,

Table 6
Parameters of methane production from sugarcane leaves augmented with KKU-MC1 in each HRT under steady-state conditions.

Parameters	HRT (days)			
	40	30	20	25
Biogas production rate (mL/L·d)	158.40 ± 12.39	240.00 ± 13.64	147.00 ± 8.49	264.00 ± 7.25
MPR (mL-CH_4/L·d)	74.86 ± 6.80	120.86 ± 3.86	72.18 ± 4.88	135.43 ± 2.15
Biogas yield (mL/g-VS)	156.32 ± 12.23	180.00 ± 10.75	73.50 ± 4.24	165.00 ± 14.90
MY (mL-CH_4/g-VS)	67.13 ± 7.00	84.83 ± 3.36	59.24 ± 2.13	82.57 ± 1.22
Methane content (%)	47.24 ± 1.45	50.36 ± 1.61	49.09 ± 0.98	51.30 ± 0.64
pH	7.14 ± 0.08	6.93 ± 0.12	6.34 ± 0.14	7.14 ± 0.05
VFA (g/L)	5.30 ± 0.35	5.20 ± 0.46	6.60 ± 0.09	5.40 ± 0.60
Alkalinity (g/L)	8.30 ± 0.17	8.40 ± 0.17	9.10 ± 0.30	8.70 ± 0.52
VFA/Alkalinity ratio	0.63	0.62	0.73	0.62
Acetic acid (g/L)	0.48 ± 0.03	0.48 ± 0.01	0.27 ± 0.01	0.45 ± 0.04
Propionic acid (g/L)	0.02 ± 0.01	0.04 ± 0.00	0.10 ± 0.03	0.02 ± 0.01
Butyric acid (g/L)	0.01 ± 0.00	0.05 ± 0.01	0.07 ± 0.01	0.04 ± 0.02
Lactic acid (g/L)	0.00 ± 0.00	0.00 ± 0.01	0.21 ± 0.01	0.00 ± 0.00

MPR: Methane production rate, MY: Methane yield, VFA: Volatile fatty acid.

30-, and 25-days HRT ranged from 6.93 to 7.14, falling within the pH range suitable for a stable methanogenic fermentation process. However, at the HRT of 20 days, a lower pH value (6.34 ± 0.14) was observed, accompanied by a relatively high VFA/alkalinity ratio (0.73) (Table 6). The short HRT leads to a high initial substrate loading rate, which could gradually induce microbial washout, thereby affecting methane production [59]. Furthermore, within the system operating under a short HRT, the acid-producing bacteria tend to grow at a faster rate compared to the methane-producing bacteria [26]. This imbalance leads to the accumulation of VFAs, resulting in a decrease in pH. Consequently, methane production is hindered and eventually ceases. The total VFA concentration in the effluent at HRT of 40, 30, and 25 days was relatively similar, ranging from 0.50 to 0.57 g/L, with acetic acid as the primary metabolite (0.45–0.48 g/L) (Table 6). In addition, minor amounts of propionic acid and butyric acid were detected. At the HRT of 20 days, the concentration of acetic acid detected in the effluent was relatively low (0.27 ± 0.01 g/L). Additionally, lactic acid was found at 0.21 ± 0.01 g/L, which was not observed in other HRTs (Table 6). These results demonstrate that a shorter HRT leads to the accumulation of lactic acid in the effluent, which results in the inhibition of methanogenesis and subsequently leads to a decrease in methane production.

These results indicate that a 25-day HRT was optimal for methane production from both sugarcane leaf hydrolysate and sugarcane leaves augmented with KKU-MC1, resulting in the highest MPR and MY. The experimental outcomes for MPR and MY align with the batch fermentation results, where methane production from sugarcane leaf hydrolysate resulted in a higher MPR (141.74 ± 3.38 mL-CH_4/L·d) and MY (88.59 ± 2.11 mL-CH_4/g-VS) compared to sugarcane leaves augmented with KKU-MC1 (MPR: 135.43 ± 2.15 mL-CH_4/L·d, MY: 82.57 ± 1.22 mL-CH_4/g-VS).

While the MY and MPR obtained from both reactors showed minimal differences, selecting the most suitable technique from an economic standpoint requires a thorough techno-economic analysis. From a practical perspective, this study recommends the bio-augmentation process over pre-hydrolysis. The bioaugmentation process involves a single reactor for methanogenesis, making it more practical, simpler and time-saving compared to pre-hydrolysis. In contrast, pre-hydrolysis prior to methanogenesis requires two separate reactors: a pre-hydrolysis reactor and a methanogenesis reactor. The pre-hydrolysis reactor involves cultivating an inoculum culture (KKU-MC1) in a PCS medium for 5–7 days before further use in the pre-hydrolysis of sugarcane leaves for 5 days. Therefore, augmenting KKU-MC1 into the methanogenesis reactor is a more efficient and straightforward process.

3.4. Diversity of microbial communities

The sludge samples under the optimal HRT of 25 days and an organic overloading of 20-day HRT from bioaumentation reactor were selected to investigate the diversity of microbial communities. Relative richness and diversity metrics, including Chao1, Pielou's evenness, Shannon index, and Simpson index, indicated that microbial diversity in the 25-day HRT was higher than that of the HRT of 20 days (Table 7). The high microbial diversity in the HRT of 25 days was associated with the favorable environmental conditions for the microorganisms. In contrat, in the 20-day HRT, the relative low microbial diversity was caused by unfavorable environments, such as low pH, as the reactor accumulated high concentrations of VFAs and lactic acid (Fig. 3b).

In terms of bacterial phylum-level abundances (Fig. 4a), *Bacteroidota* (28.91 %), *Firmicutes* (27.11 %), *Chloroflexi* (5.03 %) and *Verrucomicrobiota* (4.42 %) represented the four most abundant phyla under the optimal HRT of 25 days. Notably, the identification of *Bacteroidota* and *Firmicutes*, the hydrolytic bacteria, has been reported as commonly found in various biogas reactors [60,61]. The phylum *Firmicutes* is well-known for degrading complex carbohydrates such as cellulose and hemicellulose of plant structures [62]. The phylum *Bacteroidota* plays a role in protein degradation. However, the detectable presence of *Bacteroidota* is also associated with the production and accumulation of VFAs [63]. *Chloroflexi* and *Verrucomicrobiota* are bacterial phyla that have been reported to be dominate in the anaerobic digesters treating animal manure with an inoculation of anaerobic rumen fungi [64]. In the organic overloading of the 20-day HRT, the most abundant phylum observed was *Firmicutes* (28.60 %), followed by *Bacteroidota* (24.84 %) and *Proteobacteria* (3.29 %). *Proteobacteria* is another bacterial phylum that has been found as a dominant group in AD, correlating with the production of VFAs [63].

Regarding the archaeal phyla, *Halobacterota* were found to dominate in both HRTs of 25 and 20 days (Fig. 4a). Their abundance in the 20-day HRT (29.71 %) was higher than that of the 25-day HRT (9.89 %). This may be possible due to the 25-day HRT being restored after the organic overloading of the 20-day HRT (Fig. 3b). Methanogens are more sensitive to changes in the environment than acidogenic bacteria. In this study, the environmental change refers to the pH drop resulting from an accumulation of VFAs. It is notable that the suitable pH range for anaerobic bacteria is 5–8.5, while the optimal pH for methanogens is 6.8–7.2 [65]. In the 20-day HRT, a low pH of 6.34 ± 0.14 (Table 6) was deteacted, found to be lower than the optimal ranges for methanogens. Therefore, during the 20-day HRT, methanogenic archaea might be strongly suppressed and requir a longer time to fully recover. In addition, it is interesting to note that the decrease in the share of low abundance in *Halobacterota* under the optimal HRT of 25 days was due to the recover of

Table 7
Bacterial richness and alpha diversity community indexes.

Sample	HRT 20 days	HRT 25 days
Reads	107544	204239
Observed richness	705.00	1217.00
Chao1	705.00	1292.54
Pielou's evenness	0.67	0.73
Shannon	6.33	7.44
Simpson	0.95	0.98
Coverage	1.00	1.00

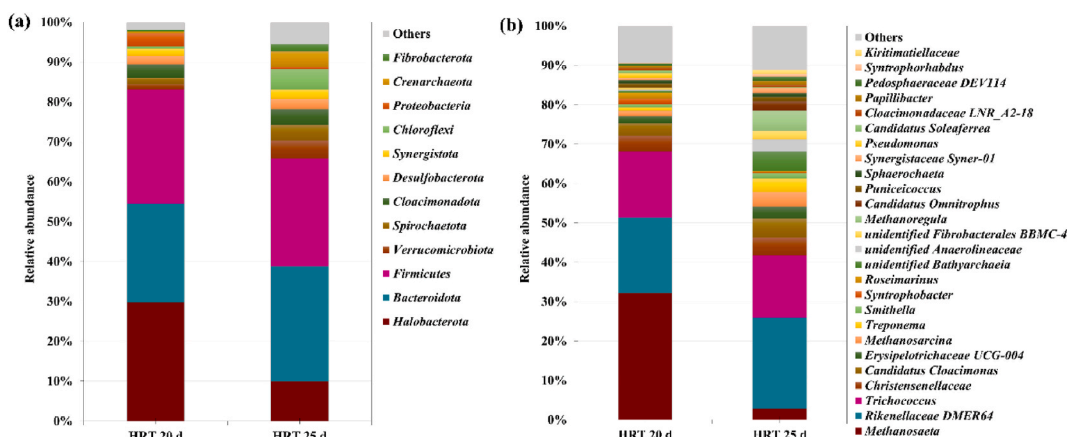

Fig. 4. The relative abundance of bacteria and archaea at the phylum level (a) and genus level (b), with taxa accounting for less than 1 % in relative abundance, were grouped and labeled as "Others."

acidogenic bacteria. This was evident from the increased abundance of various bacteria, such as *Fibrobacterota*, *Crenarchaeota*, *Chloroflexi, Defulfobacterota*, *Cloacimonadota*, *Spirochetota* and *Verrucomicrobiota*. This occurred because bacterial consortia are fast-growing microorganisms compared to methanogenic archaea, which are slow-growing microorganisms.

The genus level showed that *Methanosaeta* sp. (26.47 %), *Rikenellaceae* DMER64 (15.71 %), *Trichococcus* sp. (13.77 %), *Christensenellaceae* sp. (3.27 %), and *Candidatus Cloacimonas* sp. (2.67 %) were the most abundant in HRT of 20 days (Fig. 4b). Meanwhile, the most abundant genus in HRT of 25 days was *Rikenellaceae* DMER64 (17.23 %), *Trichococcus* sp. (11.78 %), *Christensenellaceae* sp. (3.44 %), *Candidatus Cloacimonas* sp. (3.52 %), and *Treponema* sp. (2.53 %). *Methanosaeta* sp., an acetoclastic methanogen from the *Halobacterota* phylum, exhibited a higher prevalence at a 20-day HRT (26.47 %) compared to a 25-day HRT (2.12 %), potentially due to the lower concentration of acetic acid present in the 20-day HRT (0.22 g/L) compared to the 25-day HRT (0.47 g/L). This correlation aligns with Conklin et al.'s (2006) findings, indicating that *Methanosaeta* sp. exhibits enhanced growth in conditions with lower concentrations of acetic acid. The genera *Rikenellaceae* DMER64, *Trichococcus* sp., *Christensenellaceae* sp., and *Candidatus Cloacimonas* sp. were found in both the 20-day and 25-day HRTs. These genera are associated with methane production. *Rikenellaceae* DMER64 is a mesophilic syntrophic bacterium capable of oxidizing VFAs [66] and demonstrates exceptional lignocellulosic biomass degradation abilities [67]. *Trichococcus* sp. is a fermentative bacterium capable of utilizing glucose, sugar, and lactose for the production of acids such as lactate, acetate, formate, and others [68]. *Christensenellaceae* sp. stimulates the growth of hydrolytic bacteria [69]. *Candidatus Cloacimonas* sp. exhibited the highest prevalence in biogas production using microcrystalline cellulose [70]. Additionally, this genus functions synergistically with hydrogen-consuming microorganisms and has the capacity to generate CO_2 and H_2 from proteins and amino acids [71].

4. Conclusions

This study compared lignocellulose-degrading microbes (KKU-MC1) through pre-hydrolysis and bioaugmentation for sugarcane leaf biodegradation. Pre-hydrolysis at 40 g-VS/L enhanced solubilization, but excessive substrate concentrations hindered enzyme activity and digestibility. Methane production from sugarcane leaf hydrolysate showed higher MPR with no significant difference in MY compared to bioaugmentation with KKU-MC1. In semi-continuous fermentation, optimal 25-day HRT increased methane production, while a 20-day HRT led to instability and reduced production due to VFAs and lactic acid accumulation. The microbial community structure varied with HRT, with optimal 25-day HRT favoring *Bacteroidota*, *Firmicutes*, *Chloroflexi*, and *Verrucomicrobiota*, and 20-day HRT resulting in *Firmicutes* and *Bacteroidota* dominance. This highlights the crucial role of HRT in microbial community structure. The study underscores the importance of optimizing substrate concentration and HRT for efficient sugarcane leaf methane production, offering insights into lignocellulosic biomass-to-biogas conversion for further research and process enhancement.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Napapat Sitthikitpanya: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Chaweewan Ponuansri:** Methodology, Investigation, Formal analysis. **Umarin Jomnonkhaow:** Writing – review & editing, Writing – original draft, Validation, Formal analysis, Data curation. **Nantharat Wongfaed:** Writing – review & editing, Writing – original draft, Formal analysis. **Alissara Reungsang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interest.

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