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Advancing Energy Recovery from Sugarcane Leaf via Two-Stage Anaerobic Digestion for Hydrogen and Methane Production: Impacts on Greenhouse Gas Mitigation and Sustainable Energy Production

Prawat Sukphun [1], Chaweewan Ponuansri [2], Worapong Wongarmat [2], Sureewan Sittijunda [3], Kanathip Promnuan [2] and Alissara Reungsang [1,2,4,*]

[1] Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand; prawat.s@kkumail.com
[2] Research Group for Development of Microbial Hydrogen Production Process from Biomass, Khon Kaen University, Khon Kaen 40002, Thailand; chaweewanp@kkumail.com (C.P.); wongarmat.w@gmail.com (W.W.); kanathip.promnuan@gmail.com (K.P.)
[3] Faculty of Environment and Resource Studies, Mahidol University, Nakhon Pathom 73170, Thailand; sureewan.sit@mahidol.ac.th
[4] Academy of Science, Royal Society of Thailand, Bangkok 10400, Thailand
* Correspondence: alissara@kku.ac.th

Abstract: This study aims to enhance energy recovery from sugarcane leaf (SCL) through two-stage anaerobic digestion (TSAD) for hydrogen and methane production. The influence of hydraulic retention time (HRT) on this process was investigated. Optimal conditions established through batch experiments (5% total solids (TS) (*w*/*v*) and rice straw compost inoculum) were applied in semi-continuous stirred tank reactors (CSTR-H_2 and CSTR-CH_4). Remarkably, the highest production rates were achieved with HRTs of 5 days for CSTR-H_2 (60.1 mL-H_2/L·d) and 25 days for CSTR-CH_4 (238.6 mL-CH_4/L·d). Microbiological analysis by 16s rRNA sequencing identified *Bacillus* as predominant in CSTR-H_2 followed by *Lactobacillus* and *Clostridium*. Utilizing SCL for TSAD could reduce greenhouse gas (GHG) emissions by 2.88 Mt-CO_2 eq/year, compared to open-field burning, and mitigate emissions from fossil-fuel-based power plants by 228 kt-CO_2 eq/year. This research underscores the potential of TSAD for efficient energy recovery and significant GHG emission reductions.

Keywords: biohythane; biomass; renewable energy; anaerobic digestion

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1. Introduction

The unsustainable dependence on non-renewable energy sources, such as petroleum, coal, and natural gas, has led to their rapid depletion and a surge in greenhouse gas (GHG) emissions. In response, there has been a significant push towards the development of sustainable and renewable energy alternatives. A prime example of this pursuit lies in the utilization of sugarcane leaves (SCLs), which are traditionally burned in open fields before harvesting, serving the dual purpose of enabling both manual and mechanical harvesting [1]. Residue burning accounts for 1.2 tons of CO_2 equivalent (t-CO_2 eq)/ha can cause environmental hazards. SCL is characterized as lignocellulosic biomass, primarily comprising 14.5–31.3% lignin, 9.9–25.5% hemicellulose, and 14.5–31.3% cellulose (dry weight basis) [2]. SCLs' abundance and cost-effectiveness position them as a promising substrate for second-generation energy production [3]. In this study, SCL was used as a substrate for hydrogen production.

Anaerobic digestion (AD) is one of the most effective biological treatments used for bioenergy production worldwide. AD is a series of biochemical reactions, which are mainly divided into two phases, namely the acidogenesis phase (which involves hydrolysis and

acidogenesis) and the methanogenesis phase (acetogenesis and methanogenesis). Although single-stage AD has potential in conversion of biomass to valuable resources such as methane, the implications of single-stage AD may pose problems related to unstable operation and accumulation of volatile fatty acids (VFAs), leading to process failure [4]. Two-stage anaerobic digestion (TSAD) is reported in the literature as a superior to single-stage AD in many aspects such as improving the capability of organic loading rate (OLR), reducing the retention time, minimizing the risk for process failure caused by the accumulation of VFAs, and increasing overall energy recovery by 10–40% [5,6].

One of the most important factors influencing the performance of both acidogenesis and methanogenesis phases is hydraulic retention time (HRT), which refers to the average time required for an input stream to pass through the reactor to the output port [7]. In addition, HRT is correlated with organic loading and the growth of microorganisms. Shortening the HRT has the potential to enhance the treatment efficiency of a system, but it may also lead to the instability of the reactor. Nevertheless, the limited growth of methanogens is not maintainable in reactors with short HRT, resulting in low methane production [8].

The microbial communities in the acidogenesis and methanogenesis phases differ significantly in terms of their growth conditions and requirements. In the acidogenesis phase, microorganisms are primarily involved in breaking down and acidifying organic substances present within the biomass. This process ultimately leads to the production of hydrogen as the final product. However, it is worth noting that this process has certain limitations due to its relatively low yield and conversion efficiency. These limitations can be attributed to the allocation of energy for various cellular activities and the generation of by-products such as VFAs, CO_2, and ethanol [6].

Consequently, researchers have undertaken the task of selecting inoculum sources to identify mixed cultures with complex enzymatic systems. These complex systems offer potential advantages in efficiently converting lignocellulosic biomass into hydrogen production. It is important to note that the theoretical substrate conversion efficiency into hydrogen during the acidogenesis phase is only 33%, leaving a significant portion of energy in the organic matter as VFAs by the phase's end. To address this challenge and enhance hydrogen production from lignocellulosic biomass without the need for pretreatment, various studies have explored the effects of different inoculum sources. Furthermore, for maximizing energy recovery, many researchers have taken a step further by utilizing VFAs as carbon sources for methane production [9–12]. This approach represents a promising avenue for improving the overall efficiency of bioconversion processes in renewable energy production.

Therefore, the objectives of this study are to evaluate the potential of biohydrogen in batch fermentation fed with SCL and five different inoculum sources. The effluent and residue left over from the hydrogen production were further used to produce methane. Additionally, hydrogen and methane production were investigated in a semi-continuous reactor. A 16s rDNA analysis of the microbial community during the hydrogen production stage was conducted to enhance our understanding of the relationship between microbial species and products' production at the initial and optimal HRT. Lastly, the reduced GHG emission potential of utilizing SCL for TSAD was evaluated.

2. Materials and Methods

2.1. Feedstock Preparation

The sugarcane variety KKU3 was obtained from a sugarcane plantation owned by a farmer in Ban Fang District, Khon Kaen, Thailand. The leaves were harvested during the 2021–2022 harvesting season and were approximately 12 months old at the time of harvesting. After harvesting, the leaves were sun-dried and cut by kitchen knife to 2 cm in length before being ground using a milling machine and then sieved through a 1.0 mm screen. Subsequently, they were stored in plastic containers prior to being utilized for experiments. The composition of SCL (all in %) was 38.37 cellulose, 17.57 hemicellulose,

23.80 lignin, 92.69 total solids (TS), 88.45 volatile solids (VS), and 9.88 moisture content, all on a dry weight basis.

2.2. Inoculum Preparation

The inoculum utilized for hydrogen production originated from five distinct sample sources, as follows: cow manure (CM), rice straw compost (RSC), livestock soil (LS), anaerobic digestion sludge (AS), and cow rumen fluid (RF). The inoculum preparation was conducted according to the modified method of Fangkum and Reungsang (2011) [13]. Briefly, 10 g of each inoculum source was cultivated in a 120 mL serum bottle containing 60 mL of basic anaerobic (BA) medium with 3% (w/v) of glucose as the carbon source. The BA medium consisted of (all in g/L): K_2HPO_4 0.45, KH_2PO_4 0.45, NaCl 0.9, $(NH_4)_2SO_4$ 0.9, yeast extract 5.0, $CaCl_2.2H_2O$ 0.12, $MgSO_4.7H_2O$ 0.18, $NaCO_3$ 4.0, and Cysteine HCl 5.0. The initial pH was adjusted to 7.2 using 5 M HCl or 5 M NaOH. The serum bottles were sealed with a septum and an aluminum cap before nitrogen flushing for 5 min to create an anaerobic condition. The bottles were then sterilized for 15 min in an autoclave at 121 °C. After the sterilization process, the inoculants were added to the bottles at a concentration of 10% (v/v). Then, the bottles were incubated for 7 days under mesophilic conditions (37 ± 2 °C). The inoculum was sub-cultured in fresh media at an initial inoculum concentration of 10% (v/v) and cultured for one month.

For methane production, CM mixed with RF was used as the inoculum. The inoculum was prepared by mixing CM with distilled water at 1:1 (v/v). Then, the solid residue was filtered out and the liquid was subsequently mixed with 10% rumen. The inoculum was enriched in a BA medium containing 3% (w/v) glucose as the carbon source. The fermentation process occurred under anaerobic conditions for approximately 15 days. Then, SCL mixed with BA medium was added to adjust the initial TS content to 5% (w/v). The fresh substrate was added at 10% (v/v) of the existing culture media every 15 days until the desired volume of inoculum in the fermenter was obtained. Afterward, acclimatization was performed by replacing 10% of the culture media with a fresh substrate every 15 days.

2.3. Two-Stage Anaerobic Digestion of Biohydrogen and Methane Production in the Batch Experiment

In the biohydrogen production stage, the effect of SCL concentrations and various microbial inoculum sources on the hydrogen production process was investigated. The SCL was varied at the concentration of 1.0 to 10.0% TS (w/v). The hydrogen fermentation process was carried out in 120 mL serum bottles, each with a working volume of 60 mL. Enrichment inoculum sources, namely CM, RSC, LS, AS, and RF, were added to the bottles, achieving a final concentration of 10% (v/v) with an optical density of 0.1 at a wavelength of 600 nm. The SCL concentrations varied at 1.0%, 2.5%, 5.0%, 7.5%, and 10.0% TS (w/v) for different inoculum sources. The working volume was adjusted to 60 mL using a BA medium, and the pH was adjusted to 7.2 using either 5 M HCl or 5 M NaOH. The bottles were sealed with septum and aluminum caps, and the headspaces of the bottles were purged with nitrogen gas for 5 min to establish anaerobic conditions before initiating the hydrogen fermentation process. The experiments were performed in triplicate. The bottles were incubated in an orbital shaker operating at 150 rpm and at mesophilic temperature (37 ± 2 °C). During fermentation, the biogas volume was measured using the wetted glass syringe method every 12–24 h [14]. The gas samples were collected using a gas-tight syringe for subsequent analysis of gas composition using gas chromatography (GC). At the end of fermentation, the pH of the fermentation broth was measured and recorded. Furthermore, the fermentation broth was collected to analyze the VFA composition using high-performance liquid chromatography (HPLC).

The hydrogenic effluent obtained from all experimental runs was used for the biomethane production potential (BMP) test. A 1.0% (w/v) enriched methane inoculum was added to 120 mL serum bottles containing 58 mL of hydrogenic effluent. The pH was adjusted to 7.5 using either 5 M HCl or 5 M NaOH. The bottles were sealed with a septum and an

aluminum cap. The bottle headspaces were purged with nitrogen gas for 5 min to ensure anaerobic conditions were achieved. The bottles were incubated in an orbital shaker at 150 rpm and a mesophilic temperature (37 ± 2 °C). Similar to the previous experiment, the biogas volume was measured daily using the wetted glass syringe method. The gas samples were collected daily using a gas-tight syringe for subsequent analysis of gas composition using GC. The experiments were performed in triplicate.

2.4. Semi-Continuous Setup and Operation for Two-Stage Anaerobic Digestion of Biohydrogen and Methane Production

The TSAD process consists of two CSTRs, as depicted in Figure 1. A 1.2 L CSTR with a working volume of 1 L and a 13 L CSTR with a working volume of 10 L were used for the hydrogen and methane production reactor, respectively. The headspaces of reactors were directly connected to the gas counter. The reactor was continuously stirred at 150 rpm using a magnetic stirrer with a temperature of 37 ± 2 °C controlled by recirculating heated water through a jacket throughout the experiment. The experimental procedure commenced with the hydrogen production process in the initial-stage hydrogen production reactor (CSTR-H_2). SCL was introduced into the reactor at an optimal concentration determined from batch experiments (Section 2.3). Subsequently, a 10% (v/v) inoculum was added to the reactor after adjusting its initial turbidity to 0.1 at a wavelength of 600 nm. The working volume was set to 1.0 L using the BA medium, and the pH was regulated to 7.2 by adding either 5 M HCl or 5 M NaOH. The reactor was tightly sealed, and nitrogen gas was purged into the reactor for 20 min to establish anaerobic conditions before initiating the hydrogen production process. The start-up phase of hydrogen production was conducted in batch mode for 7 days. Subsequently, when the system reached a steady state, hydrogen production rate (HPR) fluctuations not exceeding 10% of their average value, the process was shifted to a semi-continuous mode with HRTs of 5, 4, 3, and 2 days. The HRT yielding the highest HPR was the optimum HRT. The effluent was replaced with fresh media on a daily basis, in accordance with the HRT shown in Table 1. The hydrogenic effluent obtained at the optimum HRT was used as the substrate to produce methane in the second stage.

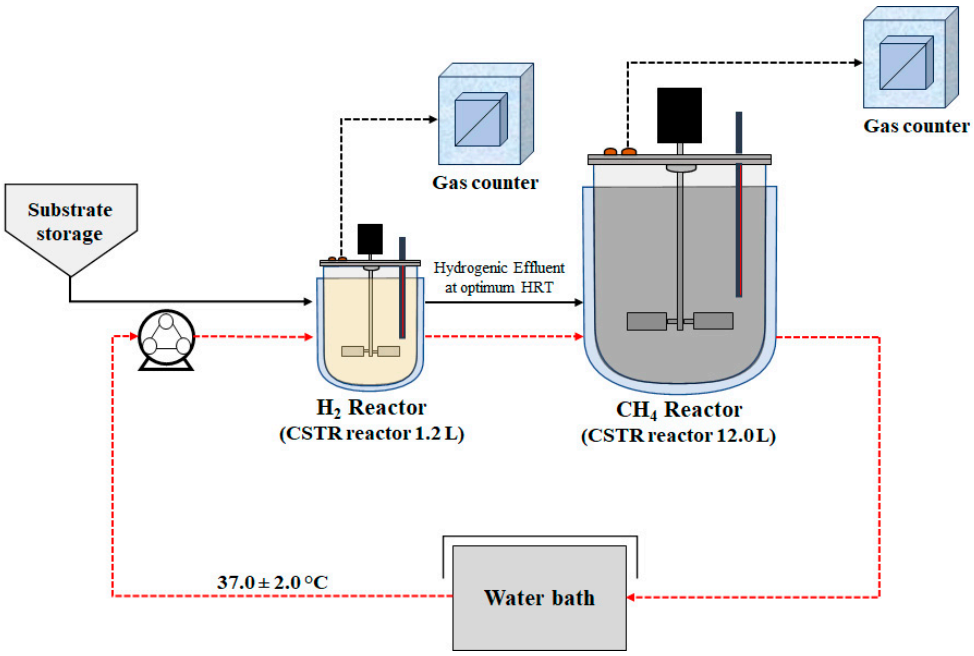

Figure 1. Process scheme for two-stage hydrogen and methane production.

Table 1. Feeding rate and volume of substrate feeding at each HRT during TSAD operation.

Runs	CSTR-H_2			CSTR-CH_4		
	Feeding Rate (L/d)	Volume of Substrate (mL)	HRT (d)	Feeding Rate (L/d)	Volume of Substrate (mL)	HRT (d)
1	0.20	200	5	0.25	250	40
2	0.25	250	4	0.33	333	30
3	0.33	333	3	0.40	400	25
4	0.50	500	2	0.50	500	20

In the start-up phase of second-stage methane production, the CSTR-CH_4 was filled with the hydrogenic effluent obtained at the optimum HRT in the hydrogen stage to obtain the working volume of 10 L. The enrichment methanogenic consortium at 1.0% (*w*/*v*) was also added. The pH was then adjusted to 7.5 using 5 M HCl or 5 M NaOH. Nitrogen gas was purged into the reactor for 30 min to create anaerobic conditions. The fermentation process in CSTR-CH_4 lasted in batch mode for 45 days. Upon reaching a steady state, the process was switched into a semi-continuous mode with HRTs of 40, 30, 25, and 20 days by feeding hydrogenic effluent as a substrate for CSTR-CH_4, as shown in Table 1.

The volume of biogas produced by both CSTR-H_2 and CSTR-CH_4 was measured and recorded using the gas counter, and samples of the biogas were collected from the gas sampling ports for analysis of composition using GC. The effluent from both reactors was also subjected to analysis for pH, the concentration of VFAs, and the VFAs/alkalinity ratio. In addition, the hydrogenic effluent from CSTR-H_2 at a steady state of initial and optimal HRT was collected to analyze the microbial community using 16S rRNA amplicon sequencing on an Illumina platform.

2.5. Analytical Methods

TS and VS content were analyzed using the standard methods [15]. The pH measurement was conducted using a pH meter (pH-500, Clean, Taiwan). To determine the VFA concentration in the fermentation broth, the HPLC (Shimadzu LC-10AD, Kyoto, Japan) equipped with a VertiSep™ (Bangkok, Thailand) OA 8 mm column and a refractive index detector (RID) was employed. The analysis was conducted under controlled conditions, specifically at a column temperature of 40 °C. The mobile phase used was a solution of 5 mM H_2SO_4, at a flow rate of 0.5 mL/min. Before conducting HPLC analysis, liquid samples underwent centrifugation at 10,000 rpm for 5 min, and the clear supernatant was collected and mixed with 0.2 M oxalic acid. The samples were filtered through a 0.45 µm cellulose acetate membrane. The external VFA standards used for HPLC calibration contained a mixture of VFAs at concentrations in the range of 1, 2, 5, 10, and 20 mM. Individual VFAs used for standards were HPLC and ACS grade (Sigma Aldrich, Massachusetts, United States). The remaining alkalinities and VFAs were analyzed according to standard methods [15]. The contents of cellulose, hemicellulose, and lignin were determined following the method described by Goering & Van Soest [16]. The composition of biogas was analyzed using a GC (Shimadzu GC-17A) equipped with a 2 m stainless steel Shin carbon (50/80 mesh) column and a thermal conductivity detector (TCD). The analysis was performed at detector, column, and injector port temperatures of 140, 120, and 130 °C, respectively, with helium as the carrier gas at a flow rate of 25 mL/min.

2.6. Bacterial Population Analysis Using 16S rRNA Amplicon Sequencing on an Illumina Platform

The amplification of bacterial and archaeal 16S rRNA gene fragments in the V3–V4 region was performed using primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCT AAT-3′). The polymerase chain reaction (PCR) products were subjected to purification using the Qiagen Gel Extraction Kit (Qiagen, Düsseldorf, Germany). The Illumina platform was employed for the analysis of the libraries generated

with the NEBNext® UltraTM DNA Library Prep Kit for Illumina. The quantification of these libraries was performed using the Qubit fluorometer. Sequence analyses were performed using the Uparse software v7.0.1001, employing all effective tags as described by Edgar (2013) [17]. Sequences exhibiting a similarity of ≥97% were allocated to the same operational taxonomic unit (OTU). The diversity of the samples was assessed through the use of the abundance-based coverage estimator Chao1, along with the Shannon and Simpson indices for diversity estimation.

3. Results

3.1. Two-Stage Anaerobic Digestion of Biohydrogen and Methane Production in the Batch Experiment

The trend of hydrogen yield (HY) of treatments that employed CM, RSC, LS, and AS as an inoculum increased with an increase in SCL concentrations ranging from 1% to 5% TS (*w*/*v*). A further rise in SCL concentrations to 7.5% and 10% TS (*w*/*v*) resulted in a drastic drop in HY for all treatments (Figure 2). The treatment that employed RSC as an inoculum showed the highest HY in the run with an SCL concentration of 5% TS (*w*/*v*), and a similar trend of decreased HY was observed when the concentration of SCL was increased (Figure 2). A maximum hydrogen yield of 19.7 mL-H_2/g-VS was attained from the treatment of RSC with an SCL concentration of 5% TS (*w*/*v*). The low HYs obtained might be due to elevated concentrations of SCL, which limited microbial access to substrates and resulted in low microbial activities. These results are consistent with those of Margareta et al. (2020), who revealed that the bacterial growth rate was inhibited at high concentrations of substrates and metabolite products [18]. Considering the results on HY from using different sources of inoculum, it was observed that the highest HY was obtained from the treatment of RSC followed by CM, LS, AS, and RF, respectively. These results indicate that RSC was rich in cellulolytic microorganisms, which have the potential to decompose lignocellulosic materials. This finding is consistent with the research by Wongfaed et al. (2023), who observed that the enriched RSC consortium exhibited the highest filter paper degradation efficiency of 51.95% (VS basis) (*w*/*v*) with a xylanase activity of 0.66 international units/mL [19]. In contrast, the degradation efficiencies of CM, soil around goat and sheep stalls (SGS), and termite intestines (TI) were lower, with values of 44.25%, 50.81%, and 43.24% (VS basis) (*w*/*v*), respectively. The maximum HY of 19.7 mL-H_2/g-VS obtained under the optimal condition of this study was slightly lower than the findings of Miftah et al. (2022), who reported that separate hydrolysis and fermentation of two-stage anaerobic digestion for hydrogen and methane production from deep eutectic pretreated SCL (4 g-VS_{added}/L) resulted in an increase in HY from 1.2 mL-H_2/g-VS to 26.7 mL-H_2/g-VS when compared to untreated SCL [20]. This result suggests that selecting a consortium with the ability to degrade lignocellulosic biomass as the inoculum source is an attractive approach to improving degradation efficiency and enhancing HY compared to chemical pretreatment.

At the end of hydrogen production, the hydrogenic effluent of all treatments was recovered and subjected to the second-stage methane production to evaluate the biochemical methane potential in the batch mode. The results showed that the methane yield (MY) increased with an increase in SCL concentrations ranging from 1% to 5% TS (*w*/*v*) of the hydrogenic effluent. However, the use of hydrogenic effluent left over from SCL concentrations greater than 5% TS (*w*/*v*) as the substrate resulted in a drastic drop in MY for all treatments (Figure 3). The treatment of hydrogenic effluent of RSC with an SCL concentration of 5% TS (*w*/*v*) gave the highest MY of 311.6 mL-CH_4/g-VS (Figure 3). The maximum MY obtained in this study was higher than in the study by Miftah et al. (2022), who reported that separate hydrolysis and fermentation of two-stage anaerobic digestion for methane production from deep eutectic pretreated SCL resulted in an increase in MY from 61.9 mL-CH_4/g-VS to 71.7 mL-CH_4/g-VS when compared to untreated SCL [20]. This difference could be attributed to variations in inoculum sources and substrate concentrations.

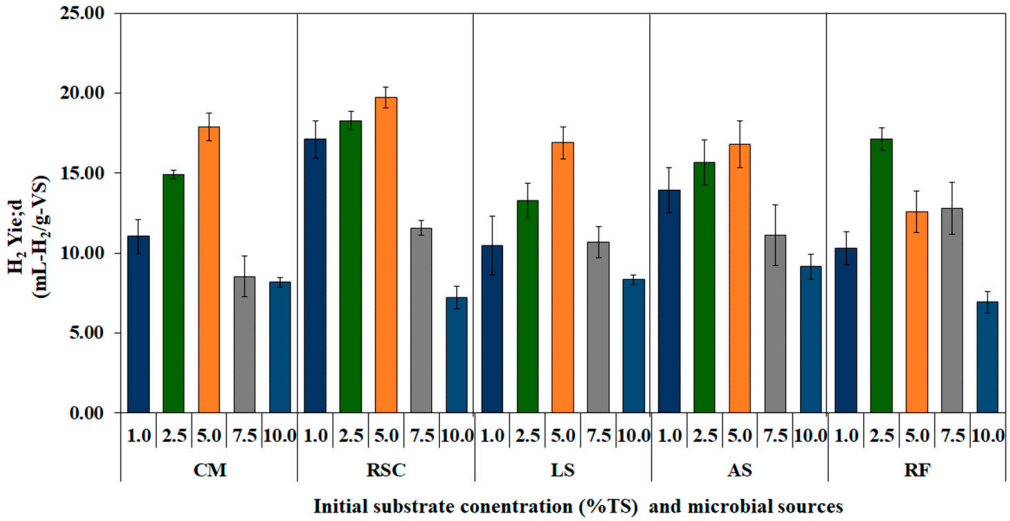

Figure 2. The hydrogen yield during the first stage of hydrogen production of 5 different inoculum sources with varying SCL concentrations. CM: cow manure; RSC: rice straw compost; LS: livestock soil; AS: anaerobic digestion sludge; RF: cow rumen fluid.

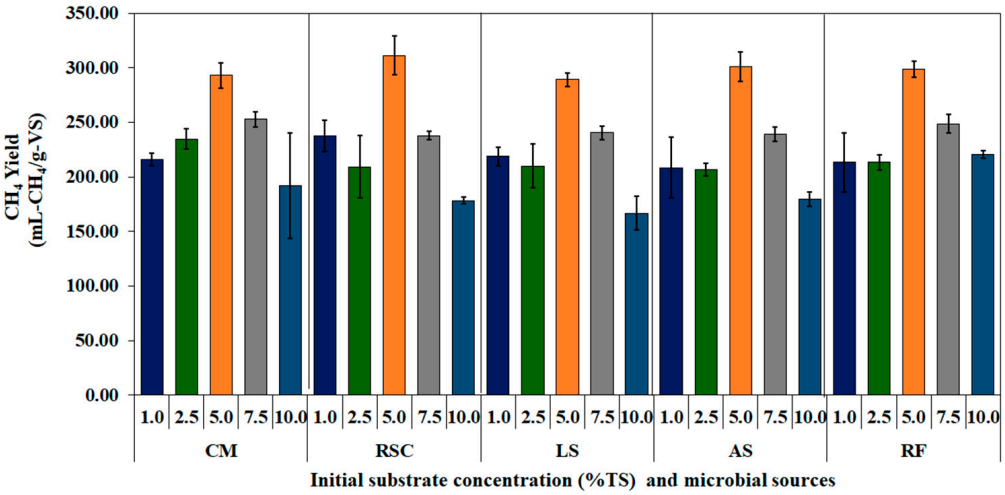

Figure 3. Methane yields during the second stage methane production of hydrogenic effluent obtained from the first stage. CM: cow manure; RSC: rice straw compost; LS: livestock soil; AS: anaerobic digestion sludge; RF: cow rumen fluid.

3.2. Effect of HRT on Biohydrogen Production Performance and Microbial Community in a Semi-Continuous Reactor

The optimum conditions from the batch experiment of 5% TS (*w*/*v*) of SCL with RSC as an inoculum were applied for the two-stage production of hydrogen and methane in a semi-continuous reactor at different HRTs. The HPR, hydrogen content, pH of hydrogenic effluent, and concentrations of soluble metabolites in the CSTR-H_2 at the different HRTs are shown in Figure 4. A stepwise decrease in HRT from 5 to 3 days resulted in an increase in HPR, with values of 11.2, 24.9, and 60.1 mL-H_2/L·d, respectively. A similar trend was observed for the hydrogen contents, which were 9.2%, 15.5%, and 19.8% at the corresponding HRTs (Figure 4a). These findings agree with Cremonez et al. (2021), who noted that a decrease in HRT is associated with higher substrate loading. This reduction in HRT to the optimal level provides sufficient substrate loading and essential nutrients for microbial consumption [7]. As can be seen in Figure 4b, the concentration of VFAs increased from 4.82 to 5.49 g/L, with acetic acid as the major component, followed by butyric and propionic acid, respectively. In theory, the process of hydrogen production is governed by thermodynamic limitations. In the presence of acetic acid, hydrogen production from

glucose follows a stoichiometric ratio of 4 moles of hydrogen per mole of glucose. In contrast, 2 moles of hydrogen per mole of glucose takes place when butyric acid is the metabolic product. Nevertheless, the production of propionic acid requires 2 moles of hydrogen [21]. However, it was observed that the pH level of the hydrogenic effluent during the HRT ranging from 5 to 3 fell within the range of 5.50–6.50, which was reported as the optimum pH range for the growth of hydrogen producers according to previous studies [22]. Additionally, the average pH of hydrogenic effluent showed a slight decrease from 5.86 to 5.70 (Figure 4c), confirming the accumulation of the VFA in the reactor.

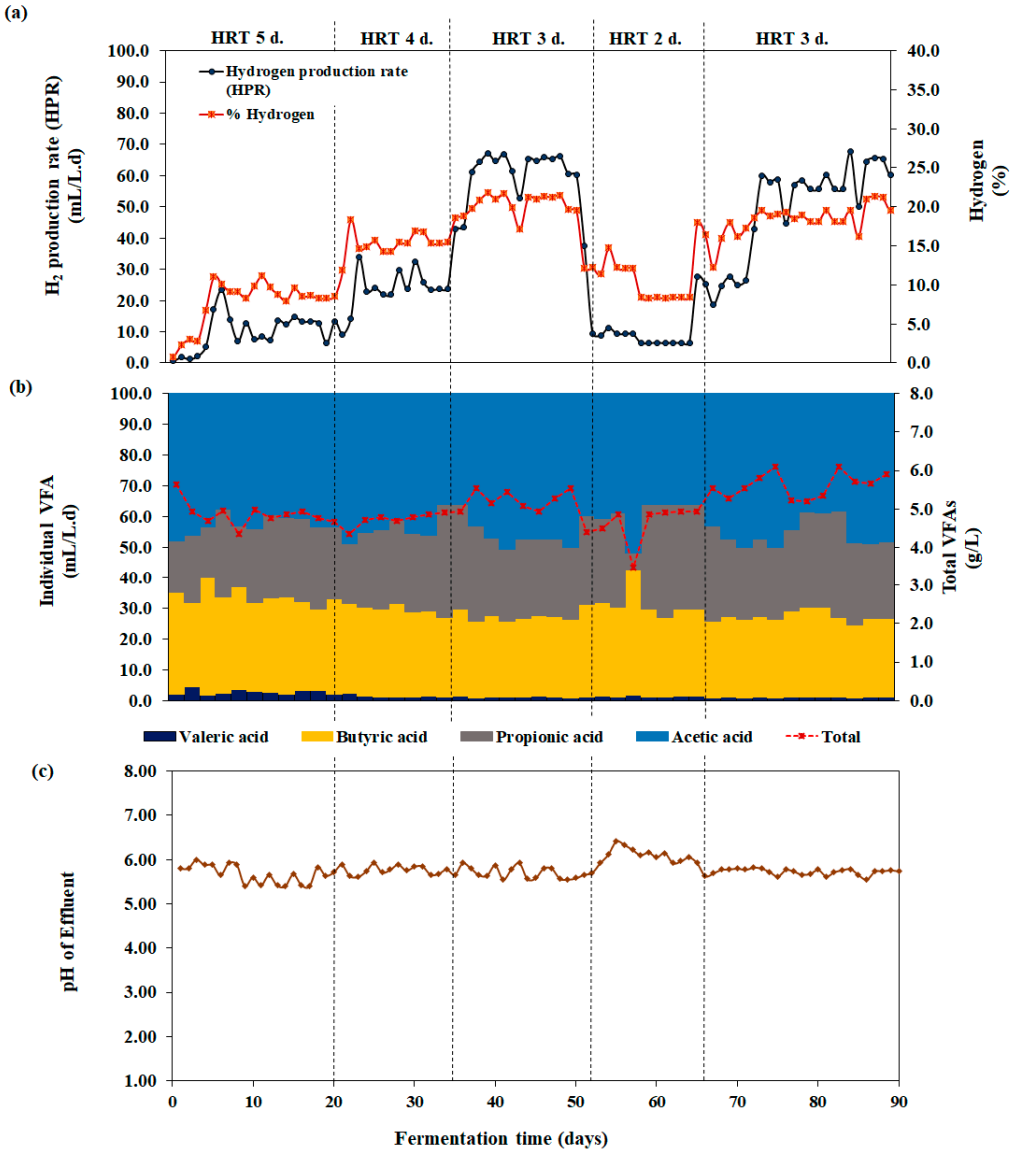

Figure 4. The profile of (**a**) HPR and hydrogen content, (**b**) concentrations of individual and total VFAs, and (**c**) pH level of hydrogenic effluent at different HRTs.

A declining trend of hydrogen content and HPR was observed when the HRT was further decreased to 2 days. The HPR and hydrogen content were 7.9 mL-H_2/L·d and 10.28%, respectively. On the other hand, the pH of the hydrogenic effluent was shifted from 5.70 to 6.10, indicating a lower hydrogen and VFA conversion rate at the HRT of 2 days. A short HRT allows microorganisms insufficient time to degrade organic substances, resulting in decreased hydrogen-producing activity. In addition, substrate overloading at a high feeding flow rate (low HRT) may result in the washout [21]. These results suggested that the optimum HRT for the CSTR-H_2 was an HRT of 3 days, resulting in a maximum HPR

of 60.1 mL-H_2/L·d, which corresponded to an HY of 5.5 mL-H_2/g-VS and a hydrogen content of 19.76%.

The monitoring of the microbial community is used to understand microbial functional changes and hydrogen production performance that occur in a CSTR-H_2 at different HRTs. *Bacillus*, *Lactobacillus*, *Acetobacter*, and *Clostridium*, with relative abundances of 23.6, 19.5, 7.3, and 2.4%, respectively, were the top four genera observed at the HRT of 5 days. At the optimal HRT of 3 days, the highest genus was *Bacillus* (26.9%), followed by *Lactobacillus* (24.0%), *Actinomyces* (8.7%), *Clostridium* (7.7%), and *Acetobacter* (6.8%), together accounting for > 50% of the community (Figure 5). These genera have been widely reported to be associated with the hydrolytic–acidogenic stage of lignocellulosic materials, which also assist in breaking down the lignocellulose complex into fermentable sugar [23]. *Bacillus* is related to producing lignin-degrading enzymes and hydrolyzing polysaccharides [24,25]. Likewise, Zabidi et al., (2020) demonstrated that *Lactobacillus plantarum* can concomitantly produce extracellular cellulolytic and hemicellulolytic enzymes such as endoglucanase, exoglucanase, β-glucosidase, and mannanase [26]. Meanwhile, *Clostridium* is a well-known hydrogen-producing bacterium with the potential to produce cellulolytic enzymes in strict anaerobic conditions. On the other hand, certain strains of *Clostridium* sp., such as *C. butyricum* and *C. tyrobutyricum,* have been reported to possess the ability to utilize lactic acid and acetic acid to produce hydrogen and butyric acid simultaneously [27,28]. Notably, *Actinomyces* is the only different species that shows up when the HRT is switched from 5 to 3 days. It has been reported to be a promising source of lignocellulolytic enzymes [29]. The relative abundances of *Bacillus*, *Lactobacillus*, and *Clostridium* increased from 23.6%, 19.5%, and 2.4% to 26.9%, 24.0%, and 7.7%, respectively, with a decrease in HRT from 5 to 3 days. These findings align consistently with a higher HPR, as shown in Section 3.2.

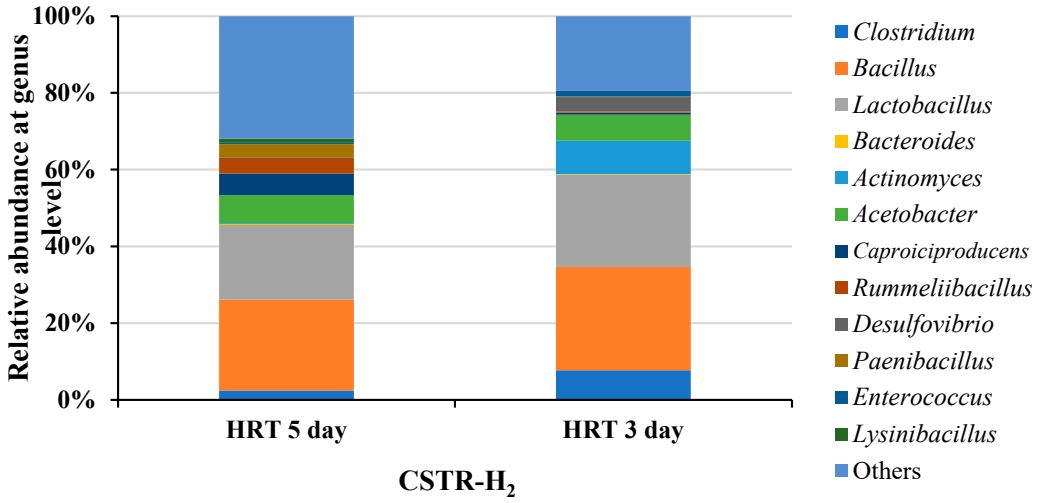

Figure 5. Relative abundances of microbial communities at genus level in CSTR-H_2 at HRTs of 5 and 3 days.

3.3. Second-Stage Methane Production from Hydrogenic Effluent in the Semi-Continuous Reactor

The hydrogenic effluent obtained from CSTR-H_2 under the optimal HRT was directly fed as the substrate for CSTR-CH_4. The MPR, methane content, and pH of methanogenic effluent in CSTR-CH_4 at the various HRTs are shown in Figure 6. A stepwise decrease in HRT from 40 to 30 and 25 days resulted in an increase in MPR. At the steady state of HRT of 25 days, the maximum methane production was observed with an MPR and methane content of 238.6 mL-CH_4/L·d and 65.84%, respectively (Table 2). A further decrease in HRT to greater than 25 resulted in a decrease in the MPR and methane content. The results indicated that an HRT of 25 days was the optimal HRT for CSTR-CH_4. The improvement in methane production when the HRT was decreased from 40 to 25 days was due to increased substrate availability. With a further decrease in HRT to 20 days, substrate overloading

was observed as both the MPR and methane content significantly reduced to 106.3 mL-CH_4/L·d and 56.54%, respectively. This reduction is caused by an imbalance between the consumption and accumulation of VFAs. At an HRT of 20 days, the pH gradually dropped to 6.72, consistent with a 1.5-times increase in the VFA/alkalinity ratio compared with the HRT of 40 days. These correlations were similar to those observed in the study by Dareioti et al. (2022), who found that at a lower HRT of 20 days, the process showed evidence of inhibition and/or overload, such as an accumulation of VFAs and a decline in MPR and MY. Moreover, the maximum MPR and MY of 0.44 L-CH_4/L·d and 295.3 mL-CH_4/g-VS added, respectively, were obtained at the HRT of 25 days [30]. Likewise, Ramos and Silva (2020) reported an increased MPR with a higher OLR achieved by reducing HRT to the optimal level in a two-stage hydrogen and methane production process from sugarcane silage in a sequential fluidized bed reactor [31]. Similarly, the study by Cabrera-Diaz et al. (2017) showed that the MPR of the two-stage methane production system of sugarcane vinasse increased from 0.43 to 2.57 L-CH_4/L·d in an up-flow anaerobic sludge blanket reactor and from 0.5 to 2.26 L-CH_4/L·d in an anaerobic packed bed reactor with the HRT decrease from 10 to 1.3 days [32].

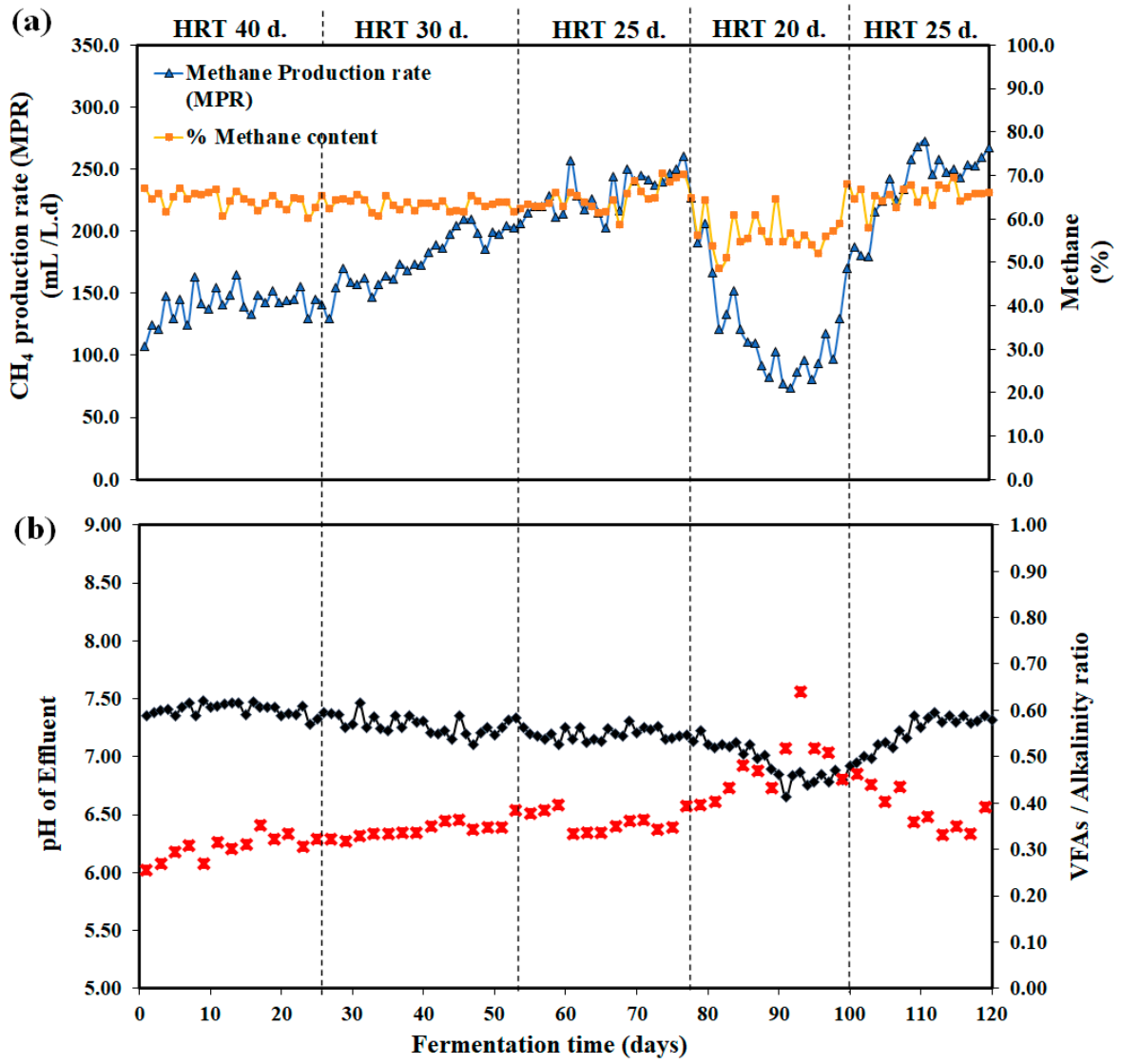

Figure 6. The profile of (**a**) MPR and methane content, and (**b**) pH level of methanogenic effluent and VFA/alkalinity ratio at different HRTs.

Table 2. Experimental data under steady state in semi-continuous sequential production of hydrogen and methane at each HRT.

Parameters	Values			
First-stage H_2 Production				
HRT (days)	5.0	4.0	3.0	2.0
HPR (mL-H_2/L d)	11.2 ± 1.0	24.9 ± 1.3	60.1 ± 6.4	7.9 ± 1.7
HY (mL-H_2/g-VS)	1.9 ± 0.7	3.2 ± 0.5	5.5 ± 0.6	0.5 ± 0.1
H_2 Content (%)	9.21 ± 0.97	15.46 ± 0.83	19.76 ± 1.48	10.28 ± 2.24
Energy recovery (kJ/g-VS) *	0.02 ± 0.01	0.04 ± 0.01	0.07 ± 0.01	0.01 ± 0.01
pH	5.86 ± 0.2	5.77 ± 0.09	5.70 ± 0.10	6.10 ± 2.24
Total VFAs (g/L)	4.82 ± 0.33	4.79 ± 0.09	5.49 ± 0.23	4.57 ± 0.53
Individual VFA (%)				
Acetic acid	41.46 ± 2.31	42.98 ± 3.77	46.44 ± 1.67	39.87 ± 5.56
Butyric acid	25.57 ± 2.84	27.71 ± 4.62	26.41 ± 1.02	28.37 ± 10.57
Propionic acid	30.19 ± 2.16	27.71 ± 4.62	26.05 ± 1.02	30.45 ± 4.82
Valeric acid	2.79 ± 0.63	1.22 ± 0.13	1.1 ± 1.02	1.31 ± 0.25
Second-stage CH_4 Production				
HRT (days)	40.0	30.0	25.0	20.0
MPR (mL-CH_4/L d)	141.1 ± 9.3	191.9 ± 25.3	238.6 ± 12.3	106.3 ± 24.8
MY (mL-CH_4/g-VS)	116.8 ± 7.4	115.1 ± 9.3	118.3 ± 22.2	42.5 ± 9.9
CH_4 Content (%)	64.02 ± 0.06	63.03 ± 1.03	65.84 ± 2.46	56.54 ± 3.59
Energy recovery (kJ/g-VS) *	4.37 ± 0.28	4.30 ± 0.35	4.42 ± 0.83	1.59 ± 0.37
pH	7.41 ± 0.06	7.25 ± 0.07	7.24 ± 0.08	6.72 ± 0.15
Total VFAs (mg/L)	2526 ± 127.6	2716.2 ± 202	5857.9 ± 330.1	3376.3 ± 282.5
Alkalinity (mg/L)	8041.7 ± 526.6	7662.5 ± 489.6	8343.8 ± 979.5	6875 ± 978.9
VFAs/Alkalinity ratio	0.32 ± 0.02	0.35 ± 0.02	0.36 ± 0.03	0.50 ± 0.07

* Energy recovery can be calculated by multiplying the H_2 yield by 0.01271 kJ/mL or the CH_4 yield by 0.03738 kJ/mL [33].

The initial VS content for each HRT was 50 g-VS/L. The final VS contents were 13.9 ± 1.0, 16.4 ± 1.4, 20.2 ± 1.7, and 27.0 ± 2.2 g-VS/L for HRTs of 40, 30, 25, and 20 days, respectively. Correspondingly, the %VS reductions were 72.2, 67.2, 59.6, and 45.9. These results imply that a longer HRT provides microbes with more time for digestion, leading to higher %VS reduction. Specifically, the transition from a 25-day to a 20-day HRT resulted in a 23% decrease in VS reduction at an HRT of 20 days (Figure 6). These findings underscore the importance of maintaining a stable second-stage methane production, which is achievable with an HRT of ≥25 days.

3.4. Estimation of Reduction in GHG and Pollutant Emissions

According to the experimental results, TSAD of SCL provides the maximum HPR of 60.1 mL-H_2/L·d, with a hydrogen content of 19.76% under an operating HRT of 3 days in CSTR-H_2. In addition, in CSTR-CH_4, the MPR reached 238.6 mL-CH_4/L·d, with a methane content of 65.84% at an HRT of 25 days. The entire process offered a total TS reduction of 60% (*w*/*v*) and gained an energy recovery of 4.5 kJ/g-VS. This result implies that TSAD is an appropriate technology for utilizing and adding value to the SCL instead of resorting to burning practices during pre-harvesting. This approach can reduce GHG and pollutant emissions from open-field burning. Furthermore, converting hydrogen and methane generated by TSAD into electricity could yield greater reductions in GHG emissions compared to electricity generation from fossil-fuel-based power plants. This study compared the reduction in GHG and pollutants emissions resulting from the utilization of SCL for TSAD with the reductions achieved through the practice of open-field burning. GHG emissions resulting from the combustion of SCL can be calculated using the equation developed by Seiler and Crutzen [34].

$$E_i = BB \times EF_i \times 10^{-3} \quad (1)$$

where E_i is air emissions of species i from open-field burning of SCL (t), BB is biomass burnt (t), and EF_i is emission factors of species i (g/kg). The equation used to calculate BB was as follows:

$$BB = BL \times BA \times CC \quad (2)$$

where BL is biomass load (t/ha), BA is harvested areas of burned sugarcane (ha), and CC is combustion completeness (unitless) [35]. The BA in Equation (2) was calculated from the following equations:

$$BA = BS/Y \quad (3)$$

$$BS = B \times P \quad (4)$$

where BS is burnt sugarcane (t) and Y is sugarcane yield per harvesting area [36]. B is the percentage of burnt sugarcane per total sugarcane production (%), and P is the total sugarcane production in 2022/2023 [37]

The BL (7.9 t/ha) and CC (64%) were chosen from the study conducted by Sornpoon et al. (2014), who observed the actual burning in 13 sugarcane plantations to determine the BL and CC values [35] (Table 3). All EFs used in this study were from the suggested EFs in the previous studies [36,38]. Assuming that only 50% of the burnt SCL in 2022/2023 was utilized for TSAD, the most prevalent pollutants, CO_2 (2.6 Mt), CO (90.1 kt), PM_{10} (12.7 kt), and $PM_{2.5}$ (9.3 kt), were, respectively emitted with about 2.88 Mt-CO_2 eq emission from SCL open-field burning (Table 4).

Table 3. Parameters for estimation of GHG and pollutant emissions.

Parameters	Values	Unit	References
Sugarcane production in 2022/2023 (P)	96.8	Mt	[37]
Percentage of burnt sugarcane (B)	64%	%	[36]
Sugarcane production per harvesting areas (Y)	69.7	t/ha	[36]
Biomass load (BL)	7.9	t/ha	[35]
Combustion completeness (CC)	0.64	-	[35]

Table 4. Pollutant and GHG emissions from 50% of burnt SCL biomass in Thailand.

Emissions	Emission Factors (g/kg)	GWP Values Relative to CO_2	Air Pollution Emissions (kt)	GHG Emissions (kt-CO_2 eq)
CO_2	1152.5	1	2589.65	2590
CH_4	3.9	25	8.76	245
N_2O	0.07	298	0.16	42
CO	40.1	-	90.10	-
NO_X	1.5	-	3.37	-
NH_3	1	-	2.25	-
SO_2	0.53	-	1.19	-
$PM_{2.5}$	4.12	-	9.26	-
PM_{10}	5.65	-	12.70	-
BC	0.73	-	1.64	-
OC	1.25	-	2.81	-

In addition to mitigating air pollutants and emissions from using SCL, the potential of using the electrical energy produced by TSAD to counterbalance CO_2eq emissions originating from fossil-fuel-based electricity generation in power plants was evaluated. The conversion of hydrogen and methane into electricity can be estimated by means of combined heat and power units (CHP). The generated electricity was compared regarding the quantity of CO_2eq emissions from fossil-fuel-based electricity generation in power plants. The calculations did not include the energy consumption during TSAD operation or losses during energy transmission. A 39% electricity conversion efficiency was used in this

study, which represents commercially available technology [39]. The electricity generation from the TSAD was determined as follows:

$$E_{el} = V \times LHV \times \eta_{el} \quad (5)$$

where E_{el} is the amount of electrical energy (kWh), V is the volume of hydrogen or methane (m^3), LHV is the lower heating value or energy density of hydrogen (3.0 kWh/m^3) or methane (5.5 kWh/m^3), and η_{el} is the electricity conversion efficiency (39%).

The total estimated electricity generation from TSAD, amounting to 517,113 MWh per year, corresponds to a 228 kt-CO_2eq/year reduction in GHG emissions compared to fossil-fuel-based power plants. This calculation is based on the emission factor of the power plant, which is 0.4401 tCO_2 eq/MWh, as provided by the Thailand Greenhouse Gas Management Organization [40]. These results indicate that electricity generated from TSAD under optimal conditions could offset the CO_2 eq emissions associated with electricity generation from power plants.

4. Conclusions

This study indicates that using RSC as an inoculum enriches the cellulolytic consortium for degrading SCL. An initial SCL concentration of 5% TS (*w*/*v*) was optimal for sequential biohydrogen and methane production from SCL in the batch mode. The semi-continuous hydrogen and methane production was conducted in the CSTR-H_2 and CSTR-CH_4. The highest HPR of 60.1 L-H_2/L·d and MPR of 238.6 L-CH_4/L·d, respectively, were obtained with a total energy recovery of 4.5 kJ/g-VS achieved at the optimal HRT of 3 and 25 days in CSTR-H_2 and CSTR-CH_4. The communities in CSTR-H_2 were analyzed through 16S rRNA sequencing, revealing that *Bacillus* was the most abundant, followed by *Lactobacillus* and *Clostridium*. Furthermore, the reduction in GHG emissions from using SCL for TSAD was estimated to be 2.88 Mt-CO_2 eq/year when compared with resorting to open-field burning. Additionally, the electricity generated from TSAD was 517,113 MWh/year, which mitigated GHG emissions from fossil-fuel-based power plants by 228 kt-CO_2 eq/year. These findings suggest that TSAD has potential in agricultural waste utilization, renewable energy production, and the mitigation of air pollution contributed by the sugarcane pre-harvesting process.

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