

Article

Cascading Biomethane Recovery from Primary and Bioprocessed Food and Corn Stover Wastes: Anaerobic Thermophilic Co-Digestion from Batch to 40 L Scale-Up

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Abstract

In this study, thermophilic anaerobic digestion (TAD, 60 °C) was evaluated as a downstream waste-to-energy step within a cascading thermophilic biorefinery using four inter-related substrates—primary cafeteria wastes (pCFWs) and primary corn stover wastes (pCSWs) were evaluated without physicochemical pretreatment. Their corresponding secondary substrates, secondary cafeteria waste (sCFW) and secondary corn stover wastes (sCSWs), were residual solids generated after thermophilic bioethanol production and exopolysaccharide production, respectively. To our knowledge, this is the first study to demonstrate sequential thermophilic valorization in which primary wastes are untreated and the remaining biotreated secondary residues are subsequently converted into biomethane, adding each step to bioeconomy. Biomethane potential was quantified to determine how substrate composition and upstream bioprocessing influence methane yield and biodegradability. In the batch, pCFW achieved the highest biodegradability (84% VS reduction) but suffered rapid acidification at higher loadings, whereas pCSW was hydrolysis-limited by lignocellulosic recalcitrance. Upstream bioprocessing (biological pretreatment) improved digestibility, with sCSW exhibiting a 1.8-fold increase in methane yield (300 L CH₄ kg⁻¹ VS) relative to pCSW. All co-digestion treatments outperformed monodigestion, with the best-performing (among the tested) sCFW:sCSW ratio of 3:1 delivering the highest methane yield (413 L CH₄ kg⁻¹ VS) and VS reduction (95.8%). Scale-up in a 40 L fed-batch reactor achieved methane productivities of 49–142 L CH₄ kg⁻¹ VS per feeding cycle, reaching stable operation after two cycles, with cumulative methane production of ~800 L CH₄ kg⁻¹ VS for secondary wastes compared to ~550 L CH₄ kg⁻¹ VS for primary wastes. Microbial analysis revealed dominance of syntrophic acetate-oxidizing bacteria (*Acetomicrobium*, 22.6%) and hydrogenotrophic methanogens (*Methanothermobacter*, 72.3%). Therefore, biologically pretreated wastes enabled higher methane recovery and improved solids destruction under thermophilic conditions and demonstrates circular conversion of wastes into renewable biomethane.

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1. Introduction

Ecological concerns related to solid waste management and fossil fuel depletion have compelled the scientific community to develop environmentally safe alternative technologies for the conversion of the abundant solid organic waste into renewable fuels. Among these, food and paper waste (FPW) and corn stover (CS) are major contributors to the organic fraction of municipal and agricultural waste streams, respectively, in South Dakota. According to the U.S. EPA (2018), paper waste (PW) makes up about 28% of all MSW generated in the United States, making it one of the largest waste streams. PW also has a high recycling rate, with nearly 67% of it being recovered each year [1]. In contrast, food waste (FW) accounts for about 22% of MSW and is one of the largest materials sent to landfills because it is poorly recovered [2]. Food waste contains a high proportion of readily biodegradable organic matter and has been successfully treated at an organic loading rate of approximately 7 g volatile solids (VS)/L/day under anaerobic co-digestion conditions [3]. This makes FW a strong candidate for methane generation when processed biologically. In addition to these MSW streams, the United States produces substantial agricultural residues such as CS. With approximately 337 million metric tons of CS harvested in the 2024–2025 season [4], CS represents the largest lignocellulosic biomass resource available. Together, PW, FW, and CS form abundant and continuously generated waste streams that can be leveraged as renewable feedstocks for bioenergy production.

Anaerobic digestion (AD) is one mature and widely adopted technology, capable of diverting organic waste from landfills and reducing greenhouse gas emissions by nearly 90%. AD also stabilizes organic waste, reduces odor and pathogens, and produces a nutrient-rich digestate that can be used as a soil amendment [5], and operates across a broad range of waste compositions. In addition, the AD system can recover up to 60–70% of the biodegradable carbon in waste as biogas and achieve methane contents of 50–70% depending on feedstock. Thermophilic AD (TAD) (operated at ≥ 50 °C) also enhances hydrolysis, increasing degradation rates by 25–50% compared to mesophilic systems, and can reduce volatile solids by 40–60% [6]. TAD also gives higher methane production rates and lower hydraulic retention times than mesophilic anaerobic digestion [6]. These features make TAD particularly attractive for the treatment of high-volume organic wastes such as paper waste, food waste, and corn stover because it supports energy recovery [7]. However, since temperature-associated sensitivity can negatively impact methanogenesis despite improved hydrolysis efficiency, the overall balance between hydrolysis, acidogenesis, and methanogenic conversion becomes critical for stable TAD performance. This challenge is aggravated where certain feedstocks may introduce inhibitory effects [8]. Substrates rich in proteins, limonene, furans, lipids, antibiotics, pesticides, metals, and other organic compounds have been reported to impair AD performance [9]. To mitigate such inhibitory impacts, co-digestion of multiple substrates is commonly employed, reducing the risk of inhibition associated with individual feedstocks [10]. In the literature, anaerobic co-digestion has been shown to enhance digestion efficiency by improving biogas/biomethane yields and promoting greater solids reduction [11–15]. Therefore, the present study evaluates the biomethane potential of primary and secondary solid wastes derived from cafeteria and corn processing industries and examines whether co-digestion can improve process stability and methane yield [16].

To advance efficient waste-to-energy conversion strategies, our laboratory previously established two high-temperature bioprocesses, one that utilized Cafeteria Food Waste (CFW) as the primary substrate and another that uses corn stover, here referred to

as corn stover waste (CSW), for bioethanol [17] and exopolysaccharide production [18], respectively. Both systems generate substantial amounts of organic-rich residual solids (i.e., secondary waste) that cannot be further metabolized by the native microbial communities involved in those bioprocesses[19]. The present study assessed the effect of co-utilizing secondary waste streams on methane production, system stability, and overall TAD performance. Additionally, various mixing ratios of secondary wastes were tested to determine the best-performing ratio combination for maximizing methane yield[20]. Microbial community analyses were also conducted to gain deeper insights into the microbiological diversity underpinning system performance. While most studies have previously investigated the anaerobic co-digestion of primary cafeteria waste (pCFW, primarily composed of food and paper waste) and primary corn stover waste (pCSW) [6,21,22], *no reports to date have explored the co-digestion of secondary cafeteria (sCFW) and secondary corn stover wastes (sCSWs) resulting from prior bioprocessing treatments*. Importantly, the novelty of this study does not rely solely on evaluating unexplored substrates, but rather on establishing an integrated thermophilic waste-to-energy framework that links upstream bioprocessing with downstream anaerobic digestion. Unlike conventional BMP studies that treat feedstocks as static inputs, this work systematically examines how prior biological processing, namely bioethanol production from cafeteria waste and exopolysaccharide production from corn stover, alters substrate composition, biodegradability, and digestion kinetics under thermophilic conditions. Furthermore, this study advances anaerobic digestion research by bridging laboratory-scale batch assays with fed-batch reactor operation, enabling evaluation of process stability, adaptation behavior, and methane productivity beyond static BMP endpoints.

2. Materials and Methods

2.1. Feedstock—Primary and Secondary Cafeteria and Corn Industry Wastes

Four related substrates were evaluated as two primary–secondary pairs. Primary Cafeteria Food—paper waste (pCFW) and primary corn stover waste (pCSW)—was the original untreated feedstocks. The pCFW that consisted of food waste–paper–waste mixture was sourced from the cafeteria at South Dakota Mines (Rapid City, SD, USA). The corresponding secondary substrates were residual solids recovered after upstream thermophilic bioprocessing of these primary materials, previously developed in our laboratory [17,18]. Secondary cafeteria waste (sCFW) was generated after simultaneous hydrolysis and bioethanol fermentation of pCFW using *Thermoanaerobacter mathranii* strain A3, whereas secondary corn stover waste (sCSW) was generated after exopolysaccharide production from pCSW [17,18]. Thus, pCFW–sCFW and pCSW–sCSW represent paired substrates before and after upstream biological valorization, respectively. The sCFW, i.e., the solid residue left after the bioethanol production, was filtered using a sieve varied from 15 to 10 mm pores (SM 200, Retsch GmbH, Haan, Germany) before being used. The sCSW, solid residue left after the exopolysaccharide (EPS) production, was washed and dried before being used.

The basic physicochemical characteristics of the four substrates and the enriched thermophilic methanogenic consortium used as inoculum are summarized in Supplementary Table S1. The analyzed parameters included total solids (TSs), volatile solids (VS), VS/TS ratio, total carbon, total nitrogen, carbon-to-nitrogen ratio, protein, fat, cellulose, hemicellulose, and lignin contents. Primary cafeteria waste (pCFW) and primary corn stover waste (pCSW) had VS/TS ratios of 89% and 98%, respectively, whereas secondary cafeteria waste (sCFW) and secondary corn stover waste (sCSW) had VS/TS ratios of 66% and 83%, respectively. The enriched inoculum contained 8.9% TS and 6.8% VS and had a C:N ratio of 3.1. The C:N ratios of pCFW and sCFW were 14 and 17, respectively, whereas

the corn-stover-derived substrates had comparatively higher C:N ratios. The methods used to determine these physicochemical properties are described in Section 2.7.

Reducing sugars, soluble chemical oxygen demand, and initial VFA concentrations was not determined during feedstock characterization. Because the substrates were predominantly heterogeneous solids, these soluble-phase parameters would depend strongly on the extraction procedure and were not considered representative of the total organic fraction used for VS-based loading. VFA concentrations were instead monitored during anaerobic digestion as indicators of acidification and process stability.

2.2. Inoculum Development

The inoculum was effluent from the Wastewater Reclamation Plant (WWRP), Rapid City, SD. The pH of the WWRP effluent was 5.8. The inoculum was developed and enriched through sub-culturing techniques described previously [6,23]. The wastewater-derived inoculum was enriched because the native microbial community was not previously acclimated to thermophilic conditions at 60 °C or to cafeteria and corn stover wastes. The defined anaerobic enrichment medium supplied essential macro- and micronutrients, maintained buffering capacity and reducing conditions, and supported the progressive selection of thermophilic hydrolytic, fermentative, syntrophic, and methanogenic populations. Repeated transfers with the target substrates were therefore performed to obtain an active and stable thermophilic methanogenic consortium, minimize the influence of residual organic matter in the original wastewater inoculum, and improve the reproducibility of the subsequent digestion experiments.

The enrichment medium contained (g/L): KH_2PO_4 (Sigma-Aldrich, St. Louis, MO, USA), 0.3; K_2HPO_4 (Sigma-Aldrich, St. Louis, MO, USA), 0.3; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma-Aldrich, St. Louis, MO, USA), 0.5; CaCl_2 (Sigma-Aldrich, St. Louis, MO, USA), 0.05; NaCl (Sigma-Aldrich, St. Louis, MO, USA), 0.1; NH_4Cl (Sigma-Aldrich, St. Louis, MO, USA), 1.0; KCl (Sigma-Aldrich, St. Louis, MO, USA), 0.3; yeast extract (Sigma-Aldrich, St. Louis, MO, USA), 0.05; and Cysteine. HCl (Sigma-Aldrich, St. Louis, MO, USA), 0.5. The medium was supplemented with 20 mM sodium bicarbonate (NaHCO_3) (Sigma-Aldrich, St. Louis, MO, USA) and 2.5 mL of a trace element solution per liter and then sterilized by autoclaving for 20 min at 121 °C and 15 psi. To maintain anaerobic conditions, sodium sulfide (Sigma-Aldrich, St. Louis, MO, USA) was supplemented to the serum bottles before inoculation at a final concentration of 0.025% (*w/v*). Resazurin (0.1% *w/v* stock; Sigma-Aldrich, St. Louis, MO, USA) was included in the basal medium at 1 mL/L as a redox indicator and was added before autoclaving. After autoclaving and cooling, serum bottles (500 mL total volume; Fisher Scientific, Waltham, MA, USA) containing 50 mL of medium were also sealed with butyl rubber stoppers (Fisher Scientific, Waltham, MA, USA) and aluminum crimps (Fisher Scientific, Waltham, MA, USA) and flushed with an N_2 : CO_2 (80:20, *v/v*) gas mixture (Matheson, Rapid City, SD, USA) for 10–15 min using sterile needles (Fisher Scientific, Waltham, MA, USA) to remove residual oxygen. Anaerobic conditions were confirmed by the complete loss of pink coloration of resazurin before inoculation.

For substrate preparation, 1 g of volatile solids (VS) from a mixture of primary corn stover waste (pCSW) and primary cafeteria waste (pCFW) in equal proportions was used. The bottles were kept at 60 °C with continuous shaking at 100 rpm in an incubator shaker (Eppendorf, Enfield, CT, USA). Once stable methane production was achieved, 10% (*v/v*) of the active culture was moved into fresh 200 mL medium containing an additional 1 g of mixed waste. After 10 serial transfers, the thermophilic methanogenic consortium was successfully enriched and could utilize cafeteria and corn stover wastes as sources of carbon and energy under 60 °C incubation conditions.

Optimization of temperature and pH for methane production from mixed wastes was carried out using a 1% (*w/v*, dry weight basis) substrate concentration. The enriched

consortium was incubated across a range of 50–70 °C to identify the optimal conditions for methane generation. Additionally, six different initial pH levels were evaluated to determine the most favorable pH for methane production (details provided in Supplementary Table S2). The optimization process guided the experiments with an initial pH of 7.5, substrate loading of 2–3% (*w/v*), and an inoculum density of 10% (*v/v*) for enhanced methane yields.

2.3. Experimental Set up for Batch Cultures

The batch experiments conducted in this study were designed as comparative TAD assays, rather than as standardized BMP tests conducted strictly under ISO or VDI guidelines. The primary objective of these batch assays was to compare relative methane production, digestion stability, and substrate response trends among primary and secondary wastes, as well as their mixtures, under identical thermophilic operating conditions. Accordingly, the batch tests were used to identify inhibition thresholds, synergistic effects during co-digestion, and substrate-specific performance differences that inform downstream reactor operation, rather than to establish absolute BMP values for regulatory certification purposes.

Batch experiments were accomplished in 500 mL serum bottles, each containing 200 mL of working volume. Following the addition of anaerobic medium, substrate, and 10% (*v/v*) inoculum, the bottles were capped with rubber septa and sealed with aluminum crimps and then purged with ultrapure nitrogen for 15–20 min to establish anaerobic conditions. The serum bottles, called digesters here, were kept at 60 °C and agitated at 50 rpm. Methane concentration in the headspace was monitored every 2–3 days over a period of 16 to 24 days, depending on the methane production rate, and measurements continued until methane generation plateaued. Control bottles containing only inoculum or only substrate were also maintained to account for endogenous methane production. Methane yield was calculated as milliliters of methane produced per gram of volatile solids (VS) loaded ($\text{L CH}_4 \text{ kg}^{-1} \text{ VS loaded}$).

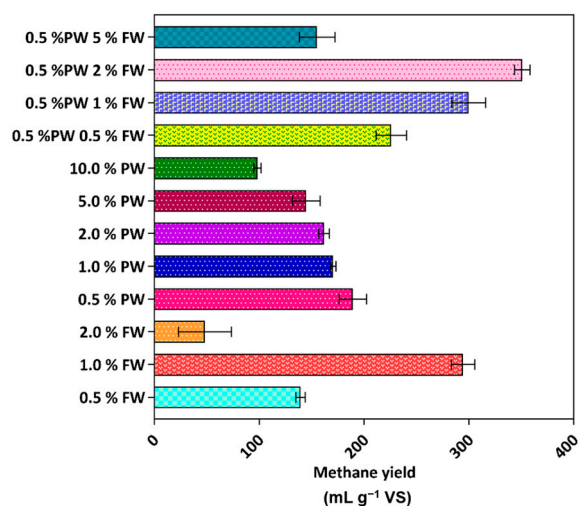
While methane yields are reported on a volatile solid basis ($\text{L CH}_4 \text{ kg}^{-1} \text{ VS loaded}$) to enable comparison with the existing literature, it is acknowledged that standardized BMP protocols typically employ lower inoculum-to-substrate ratios, longer incubation periods, and strict end-point criteria. In contrast, the present batch assays prioritize process-relevant performance indicators, including methane production kinetics, pH evolution, and volatile fatty acid accumulation, which are critical for assessing thermophilic digestion behavior and scale-up feasibility. Formal kinetic modeling was not pursued in this study, as the primary objective was to establish comparative digestion behavior and operational feasibility under thermophilic conditions rather than to develop predictive rate-based models.

2.3.1. Monodigestion of Primary and Secondary Wastes in Batch Tests

Monodigestion studies were carried out with individual primary and secondary wastes and the tests were set up as shown in Supplementary Table S3. These monodigestion batch assays were conducted to establish substrate-specific loading limits, inhibition behavior, and baseline methane production trends under thermophilic conditions. The pCFW could be segregated into two separate fractions—paper waste (PW) and food waste (FW). Different dry weight ratios of PW to FW (PW:FW = 1:1, 1:2, 1:4) were initially tested to know the optimum ratio for the highest methane yield. Since 0.5% (*w/v*, VS basis) PW gave the highest methane yield when digested individually; therefore, this concentration was used for their co-digestion with increasing concentration of FW from 0.5 to 2% (*w/v*). The PW:FW ratio giving highest methane yield was used as pCFWs in this study. Different organic loadings of pCFWs (as shown in Figure 1a) were used for the batch TAD tests.

Batch TAD tests were also conducted using pCSW at various organic loadings, 1%, 2%, 5% and 10% (*w/v*, dry weight basis). All the batch tests were conducted without a pre-treatment step as is usually seen in the case of many lignocellulosic materials [24,25]. The biomethane potential of the two secondary wastes (sCFW and sCSW) was also analyzed individually by inoculating 1% (*w/v*) dry weight and the results were compared with the methane yield from primary wastes (pCFW and pCSW).

(a)



(b)

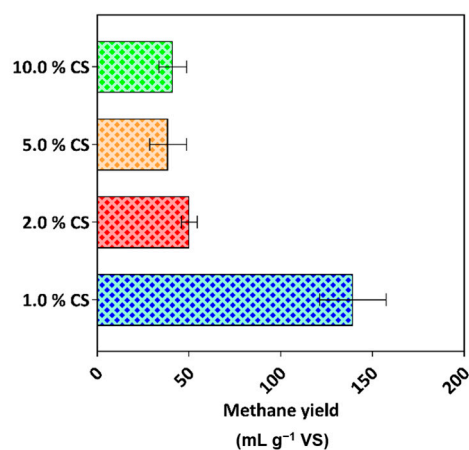


Figure 1. (a) Methane production using different organic loadings (based on volatile solids) of primary cafeteria wastes (pCFWs) (cafeteria wastes was a mixture and food and paper wastes; FW represents food waste; PW represents paper waste), and (b) methane production using different organic loadings (based on volatile solids) of primary corn stover waste (pCSW).

2.3.2. Co-Digestion of Secondary Wastes

Different dry weight mixing ratios of sCFW to sCSW (1:0, 0:1, 1:1, 1:3, and 3:1) were used for setting up co-digestion tests (Supplementary Table S4). The co-digestion batch assays were designed to evaluate synergistic or antagonistic interactions between secondary wastes, particularly with respect to nutrient balancing, VFA accumulation, and methane yield enhancement. The experimental setup used for co-digestion assays was like monodigestion tests. Batch TAD tests suffer from rapid depletion of essential nutrients and a more abrupt drop in pH as compared to the continuous AD process due to volatile

fatty acid (VFA) accumulation. This results in inhibition of methanogenesis and low methane yield. Therefore, the effect of the addition of enrichment media and pH adjustment was also analyzed for 4 different dry weight concentrations (1–8%) (Supplementary Figure S1) and resulted in notable improvement in methane production. Further, to check the feasibility of scale-up using the secondary wastes, TAD was conducted in semi-continuous reactor settings (Section 2.4.).

2.4. Scale up Using Fed-Batch Reactor

Scale-up experiments with fed-batch mode of operation were performed in a 40 L New Brunswick™ BioFlo® 510 reactor (Eppendorf North America, Enfield, CT, USA). About 1% *w/v* of the optimum mixing ratio (found by the batch tests) was used for each feeding cycle. The temperature was constant at 60 °C, and pH was only adjusted if it dropped below 5.5. Mixtures were prepared using a homogenizer (IKA Works Inc., Wilmington, NC, USA). The mixtures were divided into the required quantities for 1% *w/v* VS loading and kept at −20 °C until use. In the anaerobic reactor, to exhaust the residual organic material (biodegradable), the inoculum was vented by incubation at 60 °C in BioFlo® 510, until negligible gas production was observed. A total of 6 feeding cycles were performed, and further feeding could not be done due to depletion of the available secondary wastes. Before each feeding stage, an equal proportion of digested slurry was collected. The biogas produced was evaluated using gas chromatography (Agilent Technologies, Santa Clara, CA, USA). The slurry samples were analyzed for VFA and VS. Illumina sequencing (Illumina Inc., San Diego, CA, USA) of the thermophilic methanogenic consortium (TMC) was also conducted at the end of the experiment to analyze the microbial population actively producing methane.

2.5. Microbial Diversity Analysis of the Enriched Microbial Consortia

Microbial community analysis was performed using a liquid-phase digestate sample collected from the 40 L BioFlo reactor at the end of the sixth feeding cycle during digestion of the optimum sCFW:sCSW mixture of 3:1. This sample represented the microbial community present in the reactor at the endpoint of the fed-batch experiment and was not the original wastewater inoculum or the initially enriched inoculum. The liquid sample was collected aseptically using a 25-gauge needle to minimize the inclusion of undigested solid particles and was centrifuged at 12,000 rpm for 10 min to recover microbial cells. Genomic DNA was isolated from the resulting cell pellet using the QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's instructions. Because microbial sequencing was performed only on the endpoint liquid-phase sample, the analysis was used to identify the dominant microbial taxa associated with final reactor operation rather than to determine temporal microbial-community dynamics or establish causal mechanisms. Microorganisms strongly associated with the solid fraction may also have been underrepresented. DNA purity and concentration were determined with NanoDrop 1000 (Thermo Scientific spectrophotometer, Waltham, MA, USA). The hypervariable V4 region of bacterial and archaeal 16S *rRNA* genes was sequenced at the Research and Testing Laboratory (RTL Genomics, Lubbock, TX, USA) utilizing the universal primers 28F/388R for bacteria and 517F/909R for archaea, employing the MiSeq platform (Illumina Inc., San Diego, CA, USA). Subsequent raw sequencing data sets were analyzed using the QIIME bioinformatics pipeline.

2.6. Analytical Methods

The pH was measured using a pH meter (Thermo Fisher Scientific, Waltham, MA, USA) of each mixture was measured immediately before and after the digestion process to prevent CO₂ loss from the liquid phase during sample handling. Total solids (TSs), fat,

protein, VS, hemi-cellulose, lignin, and cellulose contents of the primary and secondary substrates were measured in accordance with the Standard Methods for the Examination of Water and Wastewater of the American Public Health Association (2005). The concentrations of VFA were measured using HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). A 5 mM sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA) solution was used as the mobile phase.

Headspace gas samples were injected out using gas tight syringes (Hamilton Company, Reno, NV, USA). The CH₄ and CO₂ content in biogas was measured with a gas chromatograph (Agilent Technologies 7890A) and fitted with a Supelco column packed with Porapak Q 80/100 mesh (Supelco, Bellefonte, PA, USA), with a TCD detector. Nitrogen was supplied as carrier gas at a flow rate of 10 mL/min. The injection, detector, and oven temperatures were set to 70 °C, 100 °C, and 50 °C (5 °C/min ramp rate), respectively. Total organic carbon, nitrogen, carbon, and phosphorus were quantified by Atlantic Microlab, Inc. (Norcross, GA, USA). Methane proportion was expressed as a percentage of the measured CH₄ and CO₂ fractions and was calculated as $\text{CH}_4/(\text{CH}_4 + \text{CO}_2) \times 100$.

Methane yield (L CH₄ kg⁻¹ VS) was determined as the amount of methane generated per gram of VS initially added to the digester, after adjusting for background methane production observed in the control setups [26]. Process stability was evaluated based on final pH and VFA accumulation, while performance was assessed through methane production and VS removal efficiency [6,23].

2.7. Statistical Analysis

All batch experiments were conducted in triplicate, and each data point portrayed in the figures represents the mean of three independent measurements for the respective chemical analyses. Statistical analysis was done using two-way analysis of variance (ANOVA) at a 95% confidence level ($p = 0.05$). The average values were statistically compared using GraphPad Prism version 7.04 (GraphPad Software, Boston, MA, USA). The analysis results are presented in the Supplementary Files.

3. Results and Discussion

3.1. Feedstock and Inoculum Characteristics

The characteristics of the feedstock (pCSW, pCFW, sCSW and sCFW) and the inoculum are presented in Supplementary Table S1. VS refers to the biodegradable fraction of total solids that can be decomposed during digestion, whereas the remaining portion is classified as fixed solids, which are non-biodegradable [27]. The analysis showed that primary wastes had higher organic content than secondary wastes, as reflected by the percent VS/TS ratio. The percentage VS/TS of pCFW and pCSW was 89% and 98%, respectively, while that of sCFW and sCSW was 66% and 83%, respectively. The enriched TMC used as an inoculum in this study had a TS content, VS content, and C:N ratio of 8.9%, 6.8%, and 3.1, respectively.

The reported optimum C:N ratio is frequently around 25, depending on digestion conditions, and the recommended range for efficient TAD performance is suggested to be 20–30 [20]. By these guidelines, the pCFW and sCFW with C:N ratio of 14 and 17, respectively, have higher nitrogen content and lower C:N ratio, whereas pCSW and sCSW have higher carbon content and higher C:N ratio than the recommended range. The organic composition of CFW also differed from that of CSW. The pCSW and sCSW contained higher amounts of hemicellulose cellulose and lignin, as compared to pCFW and sCFW, respectively. Although CSW exhibited a high organic content as indicated by its VS percentage, the majority comprised cellulose-rich compounds, which are resistant to

hydrolysis and thus constrain the initial degradation step. The CFWs (pCFW and sCFW) had higher amount of highly biodegradable organics as compared to CSWs (pCSW and sCSW), which makes CFWs more easily hydrolysable and digestible, thus having higher biogas potential than CSWs.

Comparatively high nitrogen content in CFWs (particularly in the form of protein) increases the chances of ammonia inhibition. In addition, highly biodegradable compounds in CFW lead to VFA accumulation and rapid acidification of the TAD system under high organic loadings. Both ammonia inhibition and VFA accumulation are detrimental to stable TAD operation and can lead to complete digester failure. Alternatively, sCSW has low nitrogen content and low biodegradability, and therefore complements sCFW in terms of their digestion characteristics. This analysis indicates that they have great potential to be co-digested, which ensures better conversion of these wastes to biogas and stable TAD operation. Furthermore, during the optimization process (Supplementary Table S2), it was observed that an initial pH of 7.5, substrate loading of 2–3% (w/v), and an inoculum density of 10% (v/v) significantly enhanced methane yields.

The four substrates evaluated in this study are not independent feedstocks but represent two biologically linked primary–secondary substrate pairs designed to investigate the influence of upstream bioprocessing on downstream TAD. The primary substrates, pCFW and pCSW, served as the original untreated feedstocks for bioethanol and EPS production, respectively. Their corresponding secondary substrates, sCFW and sCSW, were the residual solid fractions recovered after these thermophilic bioprocesses. Consequently, each secondary substrate retained the structural characteristics of its corresponding primary substrate while exhibiting altered chemical composition due to the selective removal or transformation of biodegradable components during the upstream process. This paired design enabled direct comparison of untreated and biologically processed materials to determine how prior valorization affected substrate biodegradability, methane production, volatile solid reduction, and digestion stability. Furthermore, evaluating the co-digestion of the two secondary substrates allowed assessment of whether these residual materials could be efficiently reintegrated into a subsequent TAD process, thereby maximizing resource recovery and supporting the development of an integrated circular biorefinery.

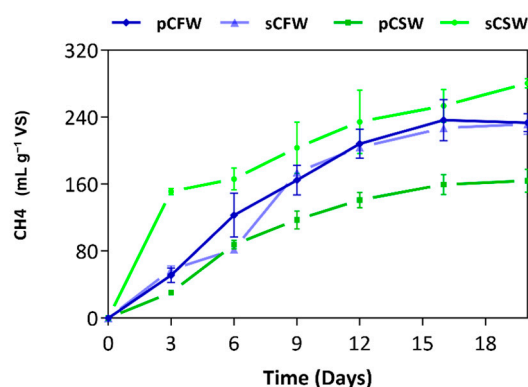
3.2. Monodigestion—TAD Performance and Stability

TAD system performance was examined in terms of methane yields and VS reduction. The methane production at different organic loadings of individual primary substrates (pCFW and pCSW) in monodigestion batch tests are shown in Figure 1a,b. This preliminary investigation was done to observe the effect of different organic loadings of the two primary wastes and to determine the optimum fraction of paper waste to food waste (PW:FW) desirable in the pCFW for maximum methane production. It is interesting to observe the synergistic effect of codigesting FW and PW, which is evident from the comparison of the batch tests (Figure 1a). Co-digestion of PW and FW mixing ratio showed higher methane yield as compared to tests where food waste or paper waste were used alone. Individual FW digestion at concentrations higher than 1% showed either significantly lower methane production (for 2% w/v FW) or no methane production (for >2% w/v FW), indicating inhibition of methanogenesis. The maximum methane yield of 351 L $\text{CH}_4 \text{ kg}^{-1}$ VS was achieved with PW:FW mixing ratio of 0.5:2, followed by 300 L $\text{CH}_4 \text{ kg}^{-1}$ VS with 0.5:1 mixing ratio. Thus, PW:FW mixing ratio of 0.5:2 was the selected composition for conducting experiments with pCFW further in this study. In addition to this, various concentrations of pCSW were also tested and the results (Figure 1b) indicated that 1% CS was the optimum concentration for methane production in batch tests, as it yielded maximum methane (139 L $\text{CH}_4 \text{ kg}^{-1}$ VS). The higher organic loadings of corn stover showed a significant decrease in methane yield.

Interestingly, FW concentrations lower than 1% (0.2% and 0.5% FW) also resulted in comparatively lower methane yields. This is likely because substrate availability at these low loadings was insufficient to sustain the methanogenic conversion rate and did not provide adequate fermentable carbon to support the metabolic needs of the active consortium. At sub-optimal substrate concentrations, although no inhibitory stress occurs, the system becomes substrate-limited rather than inhibition-limited, which explains why 1% FW, being the midpoint between substrate limitation on the low end and acidification-driven inhibition on the high end, resulted in the highest methane yield.

The major reasons for inhibition of methanogenesis at higher organic loading are the rapid VFAs or ammonia accumulation [28]. This effect is more prominent in monodigestion of food wastes as compared to paper and corn stover as it has readily hydrolysable organic carbon that accelerates the hydrolysis step, leading to VFA accumulation. It also has a high protein content, which, on digestion, forms ammonia. These intermediate product imbalances in the TAD digester cause fluctuations in the pH that adversely affect the microorganisms, methanogenic archaea being the most sensitive of them [29]. The imbalance between the various steps of the anaerobic digestion process (hydrolysis, acetogenesis, acidogenesis, and methanogenesis), if not controlled, can lead to complete system failure. Therefore, in the absence of system control in batch tests, low organic loading was preferred in this study. Figure 2a compares the methane production from the primary waste (pCFW and pCSW) with secondary waste (sCFW and sCSW) in 20 days. The cumulative methane yield and %VS reduction for primary and secondary wastes are recorded in Supplementary Table S5. Variations in VFA and ammonia concentrations can lead to shifts in pH, making pH a useful indicator of their accumulation and, consequently, of the overall stability of the digestion process. Therefore, system stability was assessed in terms of VFA production and final pH values (Figure 2b).

(a)



(b)

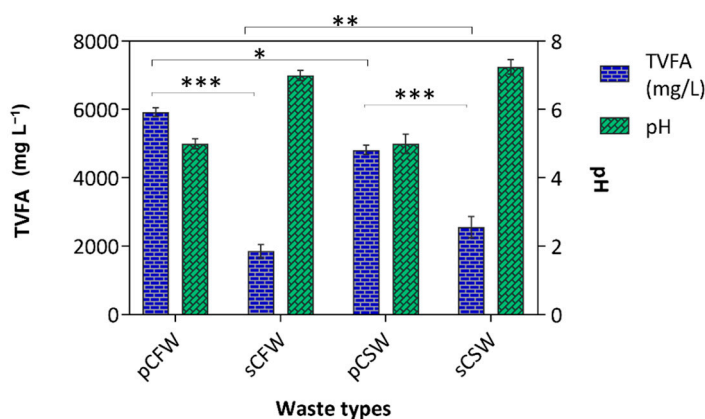


Figure 2. (a) Comparison of methane production from TAD of primary (pCFW and pCSW) and secondary (sCFW and sCSW) wastes. (b)TVFA concentration and final pH after TAD of primary (pCFW and pCSW) and secondary wastes (sCFW and sCSW). Statistical significance of p -value <0.01 , p -value <0.05 , and p -value >0.05 are denoted by ***, **, and *, respectively.

Interestingly, there was a small difference in methane yield from primary and secondary CFW, whereas this difference was significant in the case of primary and secondary CSW. The methane yield and VS reduction were slightly higher for pCFW (259.3 ± 16.9 L CH_4 kg^{-1} VS, VS reduction— $84.4 \pm 2.6\%$) when compared to sCFW (225 ± 13.4 L CH_4 kg^{-1} VS, VS reduction— $78.9 \pm 3.5\%$). This indicates that the primary and secondary CFW had similar digestibility and BMP even though secondary waste had already lost its organic carbon (mostly sugars) to the ethanol production process before TAD. The ethanol production process most likely acted as a pretreatment for hydrolyzing the complex organic compounds of CFW into easily digestible compounds. These upstream bioethanol and exopolysaccharide production steps evaluated in this study may be broadly categorized as forms of biological pretreatment, in that they modify substrate structure and composition prior to anaerobic digestion. However, unlike conventional biological pretreatments that are applied solely to enhance digestibility, these processes are designed primarily for targeted product recovery. As a result, the residual solids represent biologically conditioned feedstocks rather than intentionally optimized pretreatment outputs. This distinction is important, as it allows evaluation of whether value extraction compromises or enhances downstream methane recovery, thereby enabling an objective assessment of integrated bioprocessing efficiency.

This allowed faster conversion of proteins and lipid derivatives to methane despite the exhaustion of carbohydrates for ethanol production. In the case of CSW, sCSW gave a methane yield of 300.4 ± 11.5 L CH_4 kg^{-1} VS, which was ~ 1.8 times the methane yield obtained from pCSW (170.4 ± 21.6 L CH_4 kg^{-1} VS). The methane production correlated well with the VS reduction, which was $46.7 \pm 3.9\%$ and $72.7 \pm 4.1\%$ for pCSW and sCSW, respectively. This trend shows that the sCSW had considerably higher TAD digestibility than the pCSW possibly because the prior EPS production process hydrolyzed the recalcitrant lignocellulose to comparatively easily digestible compounds (cellulose, xylan). The effect of subjecting the wastes to bioprocesses for alternate products (ethanol and EPS) is more profound in the case of CSW as compared to CFW, mainly because TAD of lignocellulosic substrate such as CSW is limited by hydrolysis, whereas TAD of starches and sugars, such as those found in CFW, is controlled by the methanogenesis rate.

TAD performances (in context of methane yield and VS reduction) concurred with the expected system stability for each substrate, with more stable bottle reactors showing better results. Elevated concentrations of FW create conditions where acidogenesis proceeds faster than methanogenesis, leading to the accumulation of VFAs. This is evident as among the four substrates tested, pCFW showed maximum VFA concentration (~ 5925 mg L^{-1}). The pCFW utilized in this study was predominantly composed of starchy materials, which are readily hydrolyzed and assimilated by anaerobic microorganisms. This rapid breakdown can result in organic overloading, accumulation of VFAs, and a sharp drop in pH, ultimately creating conditions that inhibit methanogenesis. In line with this, the pH of pCFW dropped to ~ 5 in this study. Conversely, sCFW did not show signs of acidification, as is evident from the low VFA concentration (1858 mg L^{-1}). This is because of the shortage of rapidly hydrolyzing components (mainly carbohydrates) in sCFW, as they were consumed in the ethanol production process before TAD. The pH of this system was around neutral, which should have no adverse effect on methanogenesis, consistent with the maximum VS reduction observed for this system, indicating that the methane production was substrate-limited and not inhibited due to acidification by the end of 18 days.

Additionally, with every incremental rise in temperature, hydrolysis and acidogenesis rates accelerate more rapidly than methanogenesis, which can intensify acidification and lower system pH. Under such conditions, only a narrower subset of thermophilic methanogens can remain functional at both elevated temperature and reduced pH, which further contributes to instability if not balanced. These observations highlight the importance of managing hydrolysis–methanogenesis synchrony under thermophilic conditions. Future thermophilic TAD development may therefore benefit from sequential or partitioned reactor configurations (two-chamber systems) where hydrolytic/acidogenic processes are physically separated from the methanogenic phase to better maintain pH control and ensure sustained methane conversion efficiency.

In TAD of lignocellulosic substrates such as CSW, the primary limitation arises from the restricted availability of free water rather than organic overloading [30]. Additionally, the acidification rate of CSW is moderated by the inherently slow hydrolysis of cellulose, attributed to its recalcitrant structure and high structural integrity [31]. This slow hydrolytic process is reflected in the low methane production rates observed with pCSW (Figure 2a), suggesting that the concentration of soluble carbohydrates remained sufficiently low to render the system substrate-limited.

Methane production from pCFW, sCFW, and pCSW approached a plateau after approximately 12 days. This apparent stabilization should not necessarily be interpreted as complete degradation of all organic matter. In closed batch cultures, methane production can decline or plateau because the readily biodegradable substrate fraction becomes depleted, essential nutrients become limiting, and inhibitory intermediates accumulate [32–34]. These effects were particularly important for cafeteria-derived wastes, which contained comparatively high proportions of readily fermentable carbohydrates and proteins [35]. Rapid hydrolysis and acidogenesis therefore generated VFAs faster than they could be converted through acetogenesis and methanogenesis [36]. The resulting VFA accumulation and pH decline progressively inhibited methanogenic activity and limited further methane formation [37].

The response of sCSW differed from those of the other substrates. Methane production from sCSW continued beyond day 12 and had not completely plateaued by day 18. Corn stover contains structurally complex lignocellulosic carbon that is hydrolyzed more slowly than the readily fermentable components of cafeteria waste. However, the preceding exopolysaccharide-production process biologically conditioned the corn stover residue by partially disrupting or utilizing its lignocellulosic structure. This treatment improved the accessibility of the remaining cellulose- and hemicellulose-derived fractions without immediately solubilizing all available carbon. Consequently, fermentable intermediates were released gradually and remained available to syntrophic bacteria and methanogens over a longer period. This sustained release reduced the abrupt substrate depletion and rapid acidification observed with cafeteria-derived wastes and explains the comparatively higher and more prolonged methane production from sCSW.

The comparatively high residual VFA concentrations indicate that acidogenic conversion remained faster than methanogenic consumption in some treatments. Although the selected substrate loading and operating conditions improved methane production relative to the other conditions tested, they did not completely prevent VFA accumulation. Therefore, these conditions represent the best-performing conditions within the experimental range rather than a fully optimized state in which all fermentation intermediates were exhausted. The 18-day profiles were used as a common comparative period for the four substrates; longer incubation would be necessary to determine the ultimate methane potential of sCSW and confirm complete stabilization.

In contrast to synergistic behavior observed during co-digestion, monodigestion of individual substrates exhibited clear antagonistic effects under higher organic loadings.

Excessively rapid hydrolysis of cafeteria-derived wastes led to volatile fatty acid accumulation and pH decline, while corn stover-rich systems remained hydrolysis-limited due to lignocellulosic recalcitrance. These antagonistic responses underscore the importance of substrate balancing and provide a mechanistic rationale for the superior performance of co-digestion strategies evaluated in this study.

3.3. Co-Digestion of Secondary Wastes—TAD Performance and Stability

Comparison of the TAD batch tests using three different mixing ratios (sCFW: sCSW = 1:1, 1:3, 3:1) of secondary wastes (Figure 3a) showed enhanced methane production and this can be attributed to the synergistic effects of co-digestion [38]. The co-digestion ratios were selected as a screening design to evaluate the effect of progressively increasing the proportion of either secondary cafeteria waste or secondary corn stover waste. Monodigestion treatments, sCFW:sCSW ratios of 1:0 and 0:1, were included as controls, while 1:1 represented equal substrate contribution. The 3:1 and 1:3 ratios were selected to provide a broad and symmetrical comparison in which either substrate was present at threefold excess. These ratios were also chosen because the two substrates have complementary characteristics as follows: sCFW is comparatively nitrogen-rich and rapidly biodegradable, whereas sCSW is carbon-rich and hydrolyzes more slowly.

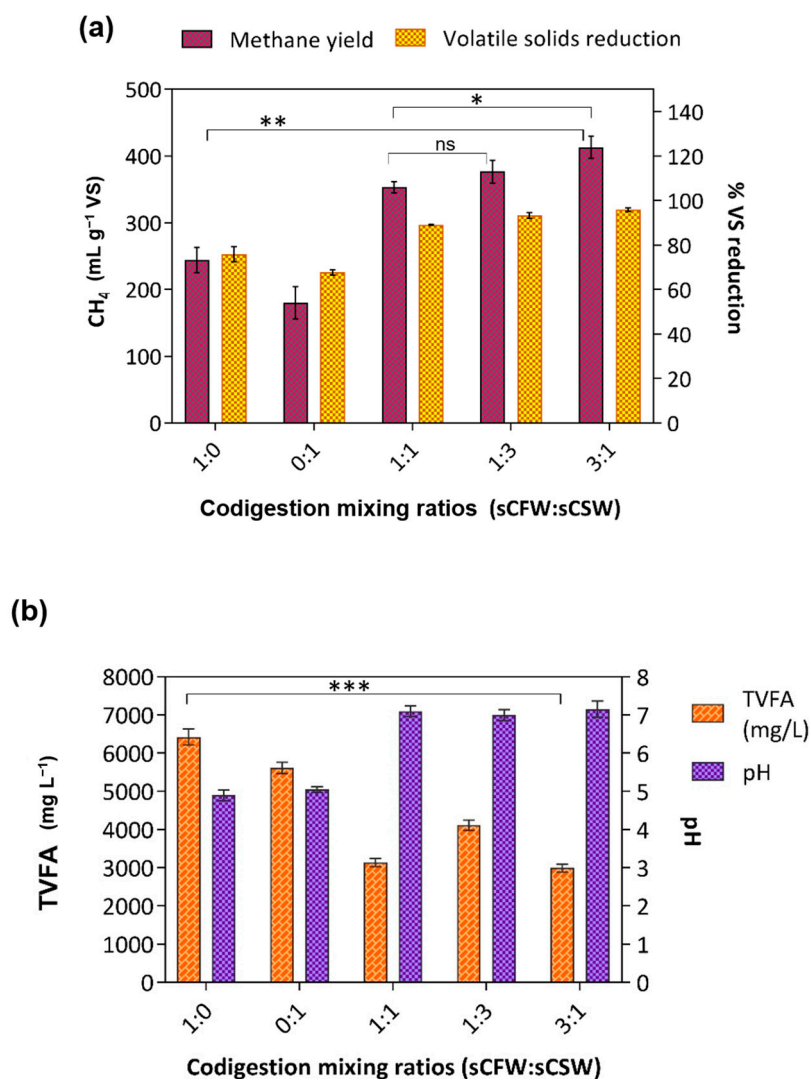


Figure 3. (a) Comparison of methane yield and VS reduction (as % of Initial VS) with different co-digestion ratios of sCFW and sCSW mixtures, and (b) TVFA concentration and final pH after TAD with different co-digestion ratios of sCFW and sCSW mixtures. Statistical significance of p -value < 0.01, p -value < 0.05, and p -value > 0.05 are denoted by ***, **, and *, respectively. “ns” represents non-significance (no p -value detected).

Evidently, all secondary waste mixing ratios outperformed ($p < 0.05$) the individual wastes, giving methane yields between 350 and 415 L CH₄ kg⁻¹ VS. In contrast, the monodigestion of secondary wastes resulted in lower methane yields, likely due to system overloading and inhibition of the methanogenic process [39]. A significant increase ($p < 0.05$) of around 20% and 28% in VS reduction was also observed for co-digestion compared to monodigestion of sCFW and sCSW, respectively. The sCFW:sCSW mixing ratio of 3:1 yielded the highest methane production and VS reduction of 413.3 L CH₄ kg⁻¹ VS and 95.8%, respectively, followed by mixing ratios 1:3 and 1:1. The C/N ratio of all the co-digestion mixtures was also closer to the recommended range (20–30) as compared to the individual wastes. This indicated that co-digestion balanced nutrients and improved biodegradability, which was translated into methane production [40,41]. Co-digestion results were also supported by the lower VFA concentrations compared to TAD of either of the individual wastes (Figure 3b).

The enhanced methane production observed during co-digestion of sCFW and sCSW reflects a clear synergistic interaction rather than simple additive behavior. This synergy arises primarily from complementary substrate characteristics as follows: sCFW provides fermentable organics and nitrogen-rich compounds that stimulate microbial activity, while sCSW contributes structurally complex carbon with lower nitrogen content, effectively moderating acidification and ammonia accumulation. By balancing hydrolysis rates and buffering volatile fatty acid formation, co-digestion mitigates the antagonistic effects observed during monodigestion of either substrate alone. The best-performing sCFW:sCSW ratio of 3:1 (among the tested) represents a trade-off point where substrate availability, nutrient balance, and mass transfer limitations are simultaneously optimized, resulting in enhanced methane yield and improved system stability.

The VFA measurement confirms that acidic conditions resulted in inhibition in assays supplied with initial loads of 2% *w/v* sCFW and 2% *w/v* sCSW (corresponding to sCFW:sCSW (VS basis) of 1:0 and 0:1, respectively) (Figure 3b). These monodigestion systems were the least stable as the pH dropped to 5 with VFA concentrations considerably higher (5500–6600 mg L⁻¹) than the co-digestion systems with sCFW and sCSW mixtures (3000–4300 mg L⁻¹). In practice, the loading rate of FW should be carefully regulated to avoid VFA accumulation and subsequent process inhibition, while the corn stover loading must be optimized to maintain adequate free water content, which is essential for effective mass transfer during digestion [30,42]. The observed link between elevated final VFA concentrations and incomplete methanogenesis in food waste systems such as sCFW aligns with findings reported in earlier studies [43,44].

Although explicit kinetic parameters were not derived using formal modeling approaches, the batch digestion assays provided critical kinetic insight through methane production profiles, stabilization time, and volatile fatty acid accumulation patterns. Differences in lag phase duration, peak methane production rate, and time to plateau among mono- and co-digestion systems reflect substrate-specific hydrolysis rates and methanogenic conversion efficiencies. In particular, the faster onset of methane production and reduced VFA accumulation observed for secondary waste mixtures indicate improved hydrolysis–methanogenesis coupling relative to primary wastes and individual secondary substrates. These qualitative kinetic indicators were used to identify stable operating windows and select feedstock combinations suitable for fed-batch operation.

3.4. Feasibility of Scale up

The transition from batch assays to fed-batch reactor operation was guided by kinetic trends observed at the laboratory scale rather than by extrapolation of absolute rate constants. Batch results informed scale-up decisions by identifying (i) organic loading ranges that avoided excessive lag phases and acidification, (ii) substrate combinations exhibiting rapid stabilization and sustained methane production, and (iii) co-digestion ratios that minimized kinetic imbalance between hydrolysis and methanogenesis. Consequently, the fed-batch experiments were designed to operate within kinetically favorable regimes identified during batch testing, allowing evaluation of microbial adaptation, process resilience, and methane productivity under repeated feeding conditions. Figure 4a,b illustrate methane production during fed-batch mode of operation in a 40 L BioFlo reactor digesting primary and secondary wastes, respectively. Specifically, these figures illustrate methane yields calculated by accounting for the cumulative methane generated during each feeding cycle relative to the amount of substrate (on a VS basis) introduced into the reactor at the start of that period. The carbon-to-nitrogen (C:N) ratios of the two feedstock mixtures, excluding the inoculum, were 20 and 24, respectively, both falling within the optimal range recommended for TAD. Through fed-batch reactor operation, the methane yield for primary and secondary waste fluctuated between 56 and 117 L Kg⁻¹ VS and between 49 and 142 L Kg⁻¹ VS, respectively. It is apparent that the co-digestion of the secondary waste led to the larger methane production in comparison with the primary wastes. Figure 5 elucidates the correlation between VFA concentration and pH at the end of each feeding cycle. Both the primary and secondary waste mixtures exhibited similar trends, where elevated VFA concentrations were linked to reduced methane yields. The pH of the reactor was stabilized between 6.8 and 7.4 at the end of each feeding cycle.

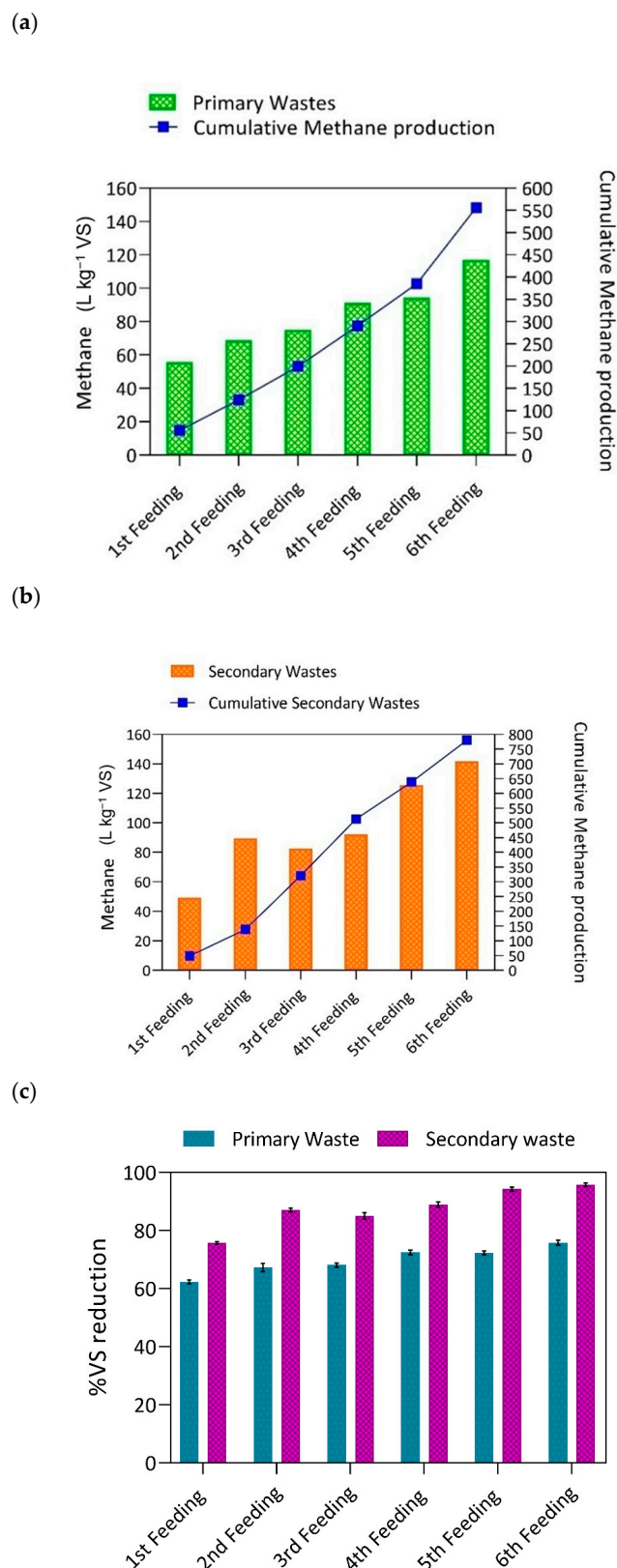


Figure 4. TAD performance in BioFlo reactor (fed-batch mode of operation). (a) Methane production using primary wastes mixtures (pCSW:pCFW = 1:1), (b) methane production using secondary wastes (sCSW:sCFW = 3:1), and (c) volatile solids reduction in each feeding cycle of primary and secondary wastes.

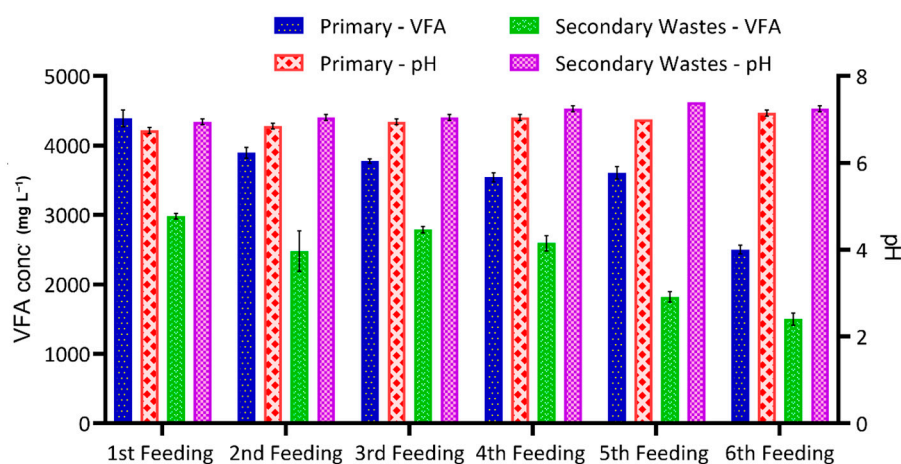


Figure 5. VFA concentration (mg L⁻¹) and pH in reactor at the end of each feeding cycle.

During the six feeding cycles, both primary and secondary waste mixture exhibited a gradual increment in methane production (Figure 4). This could be because initial feeding cycles allowed microbial communities to adjust to the environmental conditions. Initially, the pH was observed to be declining continuously, and NaOH was added to adjust pH to approximately 7.2. After the second and fourth feeding cycles of secondary and primary waste, digestions were stabilized, and pH adjustment was not needed. To evaluate the digestion performance of primary and secondary waste mixtures, the overall digestion process is segmented into two distinct stages. During Stage 1, neither culture achieved stabilization. The primary waste mixture exhibited a slightly higher average methane productivity of 73 L CH₄ kg⁻¹ VS loaded per cycle compared to 69 L CH₄ kg⁻¹ VS loaded per cycle from the secondary waste mixture. In this phase, both waste mixtures accumulated relatively high concentrations of total VFAs, averaging 3874 mg L⁻¹ and 2949 mg L⁻¹, respectively, indicating instability in the digestion systems (Figure 5). The bacterial communities actively metabolized the substrates, producing carbon dioxide and fatty acids through acetogenesis, acidogenesis, and hydrolysis. In stage 2, both primary and secondary waste cultures transitioned into stable operational conditions, resulting in improved reactor performance. Total VFA concentrations were significantly reduced to 2756 mg L⁻¹ and 2093 mg L⁻¹, respectively. Correspondingly, average methane productivity increased, reaching 106 L CH₄ kg⁻¹ VS loaded per cycle for the primary waste and 111 L CH₄ kg⁻¹ VS loaded per cycle for the secondary waste. VS reduction during digestion was comparable between the two mixtures, with stage 2 showing slightly greater VS removal efficiency than Stage 1 for both waste types. In fed-batch operation, co-digestion of secondary wastes resulted in a cumulative methane production of ~800 L CH₄ kg⁻¹ VS, representing ~45% higher methane recovery compared to primary wastes (~550 L CH₄ kg⁻¹ VS). This improvement, consistent with observations from batch tests, can be attributed to the compositional differences between the primary and secondary waste mixtures. The presence of complex lignocellulosic material in the primary waste likely impeded degradation rates, influencing both digestion performance and VS reduction efficiency. In addition to methane yield, the methane proportion of the produced biogas was evaluated to assess biogas quality. The methane content ranged from 60 to 70% (v/v) among the tested digestion systems. The highest methane proportion, 70%, was observed for sCFW, whereas sCSW showed the lowest methane proportion of 60%. The higher methane proportion observed under the 3:1 co-digestion conditions indicates more effective conversion of fermentation intermediates into methane and is consistent with the improved methane yield, lower VFA accumulation, and greater VS reduction observed for this treatment.

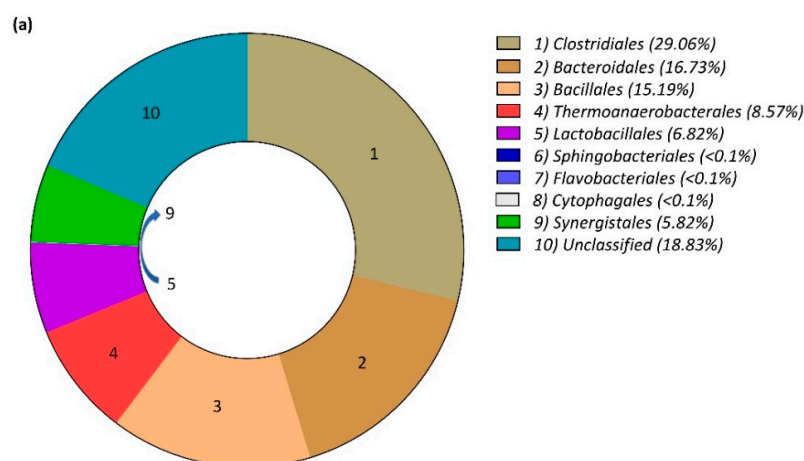
From a scale-up perspective, the transition from unstable to stable operation observed during fed-batch digestion highlights the adaptive capacity of the thermophilic microbial consortium under repeated substrate loading. While batch assays capture inhibition thresholds and synergistic trends, fed-batch operation reveals how microbial acclimation, VFA turnover, and pH buffering evolve over time—factors that govern long-term reactor performance. The faster stabilization observed for secondary waste mixtures indicates that biologically preprocessed residues reduce hydrolysis constraints and shorten start-up periods, which is a critical consideration for industrial-scale anaerobic digesters. These results suggest that secondary residues are not only suitable for scale-up, but may offer operational advantages by lowering instability risks during reactor commissioning.

It is important to note that biogas leakage occurred because of development of high internal gas pressure in the reactor and during biogas measurement. Nevertheless, higher methane productivity was achieved by secondary waste mixture and its stabilization time (time to enter stage 2) was half of what was required by primary wastes. Therefore, a longer start-up time may be needed when running large-scale, continuous digestion on such combination of primary cafeteria and corn stover wastes.

The progressive increases in methane production and VS removal during repeated feeding were attributed to the acclimation and stabilization of the thermophilic microbial consortium. During the initial feeding cycles, VFA accumulation and declining pH indicated an imbalance between hydrolysis/acidogenesis and methanogenesis. As feeding continued, active biomass was retained in the reactor and the microbial community became better adapted to the substrates and operating conditions. Consequently, VFA conversion improved, pH remained near neutral without further adjustment, and a greater proportion of the added volatile solids was converted to methane. Stabilization occurred after the second feeding cycle for the secondary-waste mixture and after the fourth cycle for the primary-waste mixture. This interpretation is supported by the lower VFA concentrations and greater methane productivity observed during the stabilized stage.

3.5. Microbial Community Analysis of the Enriched and Developed Microbial Consortia

The endpoint microbial community of the 40 L BioFlo reactor digesting the optimum sCFW:sCSW ratio of 3:1 is presented in Figure 6 (data are represented at the microbial order and genus level). The liquid-phase digestate was collected at the end of the sixth feeding cycle. Therefore, the detected community represents the dominant planktonic bacterial and archaeal populations associated with the final stage of reactor operation and should not be interpreted as a time-resolved description of microbial succession during the complete fed-batch experiment.



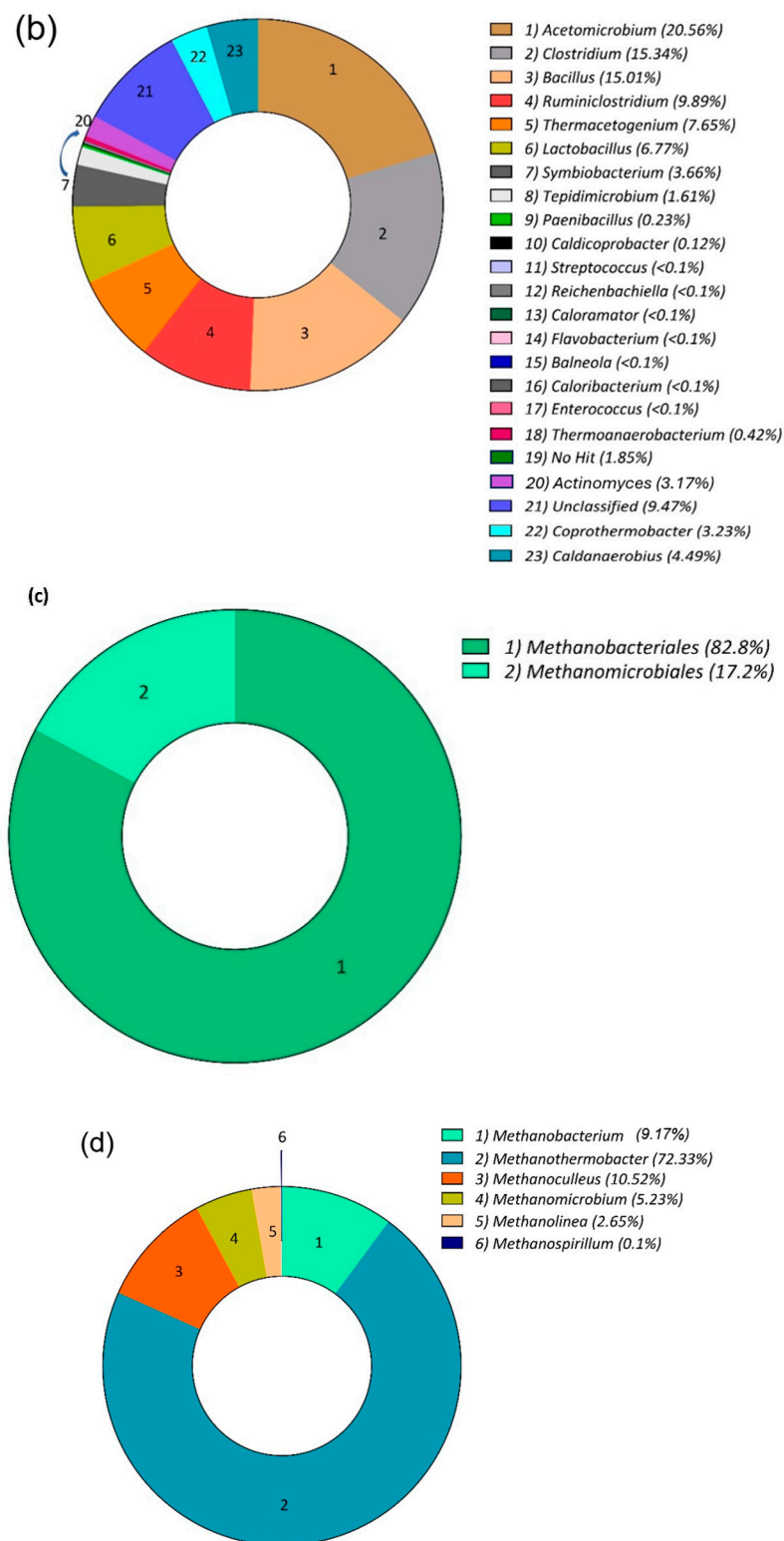


Figure 6. Relative abundance of microbial community at the order and genus levels; (a) bacteria order; (b) bacteria genus; (c) archaea order; (d) archaea genus.

The liquid samples for microbial population were collected and analyzed at the end of the reactor experiment. Analysis of the bacterial community (Figure 6a) identified *Firmicutes* (46%) and *Bacteroidetes* (16%) as major phyla. Sequences belonging to the phylum *Synergistetes* were also detected, but at low relative abundance (0.8%). Interestingly, 18.9% of the community at the family level was unclassified. Further study is needed to identify

and assign functional roles to these unclassified microbes. Within the *Firmicutes*, orders *Bacillales* and *Clostridiales* were dominant. Firmicutes are known to contain the largest number cellulolytic bacterial species [45]. As expected, the order *Clostridiales* represented highest relative abundance (39%) among the bacterial communities of TMC, followed by *Bacteroidales* (12.7%), *Bacillales* (10.2%), and *Thermoanaerobacteriales* (7.6%). The genus *Clostridium* and *Ruminiclostridium* were the two major genera belonging to the order *Clostridiales*. The dominance of the order *Clostridiales* can be attributed to their diverse roles (e.g., hydrolysis and acidogenesis) in a functional niche [46]. The microbial diversity profile strongly reflected the thermophilic nature of the system, as the archaeal population was dominated by thermophilic hydrogenotrophic methanogens such as *Methanothermobacter*, which are well-adapted to operate efficiently under high temperature conditions (55–65 °C) [47]. This clearly indicates that both the bacterial and archaeal communities were enriched towards thermophilic functionality, supporting sustained methane generation under elevated TAD operating conditions.

The first step of the TAD process involves the degradation of complex polymers (proteins, carbohydrates, and fats) to monomers and subsequently to organic acids. Therefore, the presence of FW in the reactor bottles favored the growth of carbohydrate- and protein-fermenting bacteria belonging to the order *Clostridiales*, *Bacillales*, and *Bacteroidales* [48,49]. While the genus *Bacillus* under *Bacillales* is reported to ferment starchy substrates [50], *Clostridium* and *Ruminiclostridium* under *Clostridiales* are known cellulose degraders [51–54]. Their presence can be directly correlated to their physiological roles in the digester treating FW and LWs. Numerous studies have shown that members of the order *Thermoanaerobacteriales* are capable of fermenting complex carbohydrates, including cellulose, starch, and simple sugars for H₂ production [55–57], thus allowing hydrogenotrophic methanogenesis.

Among the *Clostridium* sp., *C. cellulosi* had highest relative abundance (10.78%). Enhanced methane production from FW and LW wastes can be attributed to the presence of the cellulose-degrading bacteria [58]. Additionally, the phylum Bacteroidetes, particularly the orders *Bacteroidales*, *Flavobacteriales*, and *Cytophagales*, was found to dominate in reactors fed with cellulose and long-chain fatty acids, suggesting a key role in the breakdown of proteins, fats, cellulose, and other polysaccharides [59], which is also true for the TMC used in our study. Previous studies have also shown the dominance of the above mentioned bacterial populations in the presence of cellulosic substrates as follows: Cassava Dregs and Big manure [60], switch grass [61], corn starch [62–64], wheat straw [65], rumen of cows [66] and manure digesters [67].

At the genus level, acetogenic bacteria belonging to genus *Acetomicrobium* dominated the bacterial population (20.6%) (Figure 6b). The next abundant genus was *Bacillus* (15.3%) and *Clostridium* (15%), followed by *Ruminiclostridium* (9.9%), *Thermoacetogenium* (7.6%), and *Lactobacillus* (6.8%). The abundance of the genus *Acetomicrobium* indicates a high potential for acetate production [50]. In addition to *Acetomicrobium* sp., *Lactobacillus* sp., belonging to the order *Bacillales*, is another important acid-producing genus while *Thermoacetogenium*, belonging to the order *Thermoanaerobacteriales*, are syntrophic acetate-oxidizing bacteria. To date, only a limited range of syntrophic acetate-oxidizing bacteria have been isolated and characterized, including the thermophilic genus *Thermoacetogenium* [68]. After *Thermoacetogenium*, the next most abundant genus in the order *Thermoanaerobacteriales* were *Caldanaerobius* (4.5%, particularly *Caldanaerobius zeae* sp) and *Coprothermobacter* (3.2%). While *Caldanaerobius zeae* is polysaccharolytic, capable of fermenting wide range of sugars [69], *Coprothermobacter* are known for their proteolytic activity and coexistence in syntrophic association with methanogens belonging to genus *Methanothermobacter* [70]. *Coprothermobacter* spp. are not able to hydrolyze complex proteins but

require partially degraded substrate and are therefore better described as peptide-fermenting bacteria [71].

Among the archaeal population (Figure 6. c, d), *Methanothermobacter* (72.3%), *Methanoculleus* (10.5%), *Methanobacterium* (9.17%), and *Methanomicrobium* (5.2%) were the dominant genus belonging to two main orders *Methanobacteriales* and *Methanomicrobiales*. Ghasimi et al. also reported *Methanothermobacter* as the most dominant genus in a thermophilic reactor (50–60 °C, relative abundance >90%) [59]. While *Methanothermobacter*, *Methanobacterium*, and *Methanomicrobium* carry out hydrogenotrophic methanogenesis, few species of *Methanobacterium* can utilize acetate and butyrate as well [72,73]. *Methanothermobacter* species utilize hydrogen or formate as electron donors, thereby maintaining a low hydrogen partial pressure that supports the thermodynamic feasibility of the overall fermentation process [74]. These methanogens are characterized by relatively rapid growth, with doubling times ranging from 2 to 5 h, and are readily cultivable under laboratory conditions [59]. Alternatively, *Methanoculleus* can use ethanol and some secondary alcohols to produce methane. Evidently, hydrogenotrophic methanogens dominated the TMC in this study. These findings are aligned with previous reports showing that thermophilic conditions favor the hydrogenotrophic methanogenesis pathway [59], and the methanogens in the thermophilic reactors belonged to the genus *Methanothermobacter*. On the contrary, previous studies on AD of lignocellulosic substrates under mesophilic conditions have shown the significance of acetate utilizing methanogens, with *Methanosaeta*, *Methanosphaerula*, *Methanospirillum*, and *Methanosarcina* representing the most dominant archaeal genera [59,62,64,75,76].

The high abundance of the syntrophic genus *Acetomicrobium* (22.6%) among bacteria, along with the presence of *Methanothermobacter* (72.3%) in the archaeal community, indicates an active syntrophic acetate oxidation pathway linked with hydrogenotrophic methanogenesis [77]. This combined methanogenic pathway has also been identified by previous studies as the predominant metabolic route under thermophilic conditions [78–80].

4. Conclusions

This study demonstrates that thermophilic anaerobic digestion can effectively extend the value chain of cafeteria and corn stover wastes beyond primary product recovery. The untreated primary substrates were converted directly to biomethane without physico-chemical pretreatment, while their biologically processed residues remained highly suitable for downstream digestion. In particular, prior exopolysaccharide production improved corn stover digestibility, increasing methane yield from approximately 170 to 300 L CH₄ kg⁻¹ VS. Co-digestion further overcame the contrasting limitations of the individual substrates by balancing the rapidly degradable cafeteria residue with the more recalcitrant corn stover residue. The best-performing (among the tested) sCFW:sCSW ratio of 3:1 achieved 413 L CH₄ kg⁻¹ VS and 95.8% VS reduction, with improved pH stability and lower VFA accumulation than monodigestion. The successful 40 L fed-batch operation confirmed that these gains were retained under repeated loading, with secondary wastes delivering approximately 45% greater cumulative methane recovery than primary wastes. Overall, the findings establish a cascading thermophilic biorefinery in which cafeteria waste and corn stover are first converted into bioethanol or exopolysaccharides and the remaining residues are subsequently transformed into biomethane. This integrated strategy increases total resource recovery, reduces residual waste, and offers a practical route toward scalable circular valorization of municipal and agricultural biomass.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fermentation12080360/s1>, Figure S1: Effect of addition of enrichment media and pH adjustment on methane yield; Table S1: Characteristics of inoculum and

feedstock; Table S2: Optimization of influential parameters; Table S3: Substrate compositions in monodigestion batch tests; Table S4: Substrate compositions in co-digestion batch tests; Table S5: Cumulative methane yield and volatile solid reduction (% VS) in monodigestion tests using primary (pCFW and pCSW) and secondary wastes (sCFW and sCSW).

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