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Enhancing Biogas Production Through the Co-Digestion of Fish Waste (FW) and Water Hyacinth (WH) Using Cow Dung as an Inoculum: Effect of FW/WH Ratio

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Abstract: The current investigation explores biogas production from water hyacinth (WH) and fish waste (FW) with cow dung (CD) as an inoculum source in two scenarios. In the first scenario, the optimization of mono-digestion was performed where the effect of WH/FW (substrates) with CD (inoculum) in varied ratios of 1:1, 1:2, 2:1, and 3:1 was observed to enhance the biogas production. In the second scenario, the optimization of co-digestion using both FW and WH as substrates in different ratios (1:1, 1:2, and 2:1) with a fixed amount of inoculum was studied. The experiments were conducted in 500 mL digesters in duplicate under mesophilic conditions. Under mono-digestion conditions for FW, the digester operating with FW/CD in a 1:2 ratio demonstrated the highest biogas yield of 970 ± 14.1 mL/g VS, containing 610 CH_4 mL/g VS, while in WH, the WH/CD ratio of 1:1 exhibited the highest biogas yield of 925 ± 49.4 mL/g VS, with a methane content of 440 CH_4 mL/g VS. The co-digestion of the WH/FW ratio (1:1) showcased the highest biogas production of 1655 ± 91.92 mL/g VS, accompanied by 890 ± 70.7 CH_4 mL/g VS. This was followed by the 1:2 and 2:1 ratio, yielding 1400 ± 56.5 and 1140 ± 169.7 mL/g VS. of biogas and 775 and 585 CH_4 mL/g VS, respectively. The CD and WH mixture at a 1:1 ratio demonstrated the most significant decrease in chemical oxygen demand (COD), reaching 91.68%. COD reductions over 80% in all combinations were observed in all instances. Anaerobic digestion (AD) simulations were validated using the Gompertz model, with high correlation coefficient values (R-squared) above 0.99 for all of the studied ratios, depicting a significant correlation between experimental data and model predictions. The propionic to acetic acid ratio did not cross the threshold level, indicating no inhibition of methane production. ANOVA analysis of biogas production between the co-digestion and mono-digestion of substrates showed non-significant results ($p > 0.310$ and $p > 0.824$, respectively), while overall digestion was significant ($p < 0.024$), indicating efficiency variations among substrates. Paired sample t-tests revealed substantial differences between co-digestion ratios, which were also significant.

Keywords: aquatic macrophyte; volatile fatty acids; chemical oxygen demand; bio-waste; propionic acid/acetic acid; ANOVA

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1. Introduction

AD often demonstrates suboptimal efficiency in biogas production, whereas anaerobic co-digestion (AcoD) has enhanced biogas production efficiency [1]. AcoD enhances the degradation of resistant, complex polymeric substrates, thereby improving biodegradability. It accelerates the start-up phase, boosts overall process efficiency, and helps to regulate pH levels. Additionally, AcoD balances the carbon-to-nitrogen (C/N) ratio and stabilizes the

digestion process, which collectively maximizes productivity and enhances the efficiency of organic fraction conversion [2–4]. ACoD also diminishes the impact of inhibitors and toxins, provides essential nutrients for microbial growth, shortens retention and lag times, and enhances both the loading rate and methane production [5]. Determining the optimal mixing ratio of substrates, as well as the inoculum-to-substrate ratio (ISR), is crucial for preventing the accumulation of volatile fatty acids (VFAs) and other inhibitors that can negatively impact the AcoD process and thus reduce the biogas potentiality [6]. Various studies have reported differing results regarding the influence of ISR on biogas and methane production during the AcoD of different feedstocks. Optimizing these parameters is essential for enhancing AcoD efficiency and maximizing biogas yields [7–9]. Anaerobic co-digestion of biological wastes serves as an effective substrate for biogas production, presenting a viable waste-to-energy technology. This process offers a straightforward approach to simultaneously address the growing demand for renewable energy sources and mitigate waste management challenges [10]. Biological waste, which can serve as a substrate for anaerobic co-digestion, includes a wide range of organic materials such as crop residues, agricultural residues [11], animal manure, sewage sludge, food waste [12], fish waste [13], etc.

The Blue Revolution in India demonstrated the importance of the fisheries and aquaculture sector. The sector is considered as a sunrise sector and is poised to play a significant role in the Indian economy in the near future. India ranks as the third-largest fish producer globally and the second-largest in aquaculture, second only to China [14]. In India, over 4 million metric tons of this waste are generated annually and are often disposed of in the environment, including landfills and water bodies. The amount of waste produced during fish processing ranges from 20% to 60%, depending on the species [15,16]. In the recent past, Indian fisheries have witnessed a paradigm shift from marine-dominated fisheries to inland fisheries, with the latter emerging as a significant contributor to fish production from 36% in the mid-1980s to 70% in the recent past. Within inland fisheries, a shift from capture to culture-based fisheries has paved the way for a sustained blue economy [14]. From 2014–2015 to 2018–2019, India's fisheries sector grew by 10.88% annually. Over the past five years, India's fish production has grown by 7.53% annually, reaching 137.58 lakh metric tons in 2018–2019 to 220 lakh metric tons by 2024–2025, with an annual growth of about 9% in fish production [17].

India has around 2.36 million hectares of tanks and ponds, where culture-based fishery is predominant and contributes to the maximum share in total fish production. The current production from tanks and ponds is 8.5 million MT. As a significant contributor towards production, the department has prioritized to expand the horizontal area under tanks and ponds to achieve a target production of 13.5 million MT [14]. One of the essential items in freshwater aquaculture is the water requirement, either in hatcheries or in grow-out systems. It is estimated that freshwater aquaculture is at present utilizing 9.5 billion m^3 of water under the existing 0.745 million ha pond area, which is expected to increase to 36.2 billion m^3 at the complete development of the remaining 1.755 million ha of existing water bodies. An additional creation of 25% of new ponds, i.e., 0.439 million ha, will be achieved, thus considering the water availability of 70–80 mhm. A share of 5% (3.6 mhm) must be available for fresh aquaculture [18].

In addition, the water requirements for fish processing operations generate potentially large quantities of waste and by-products from inedible fish parts and endoskeleton shell parts from crustacean peeling process, viz., particles of flesh, skin, scales, bones, visceral mass (viscera, air bladder, gonads, and other organs), head, fins, shells, or liquid stick water. Depending on the species processed, the solid wastes make up 30–40% of the total production [19]. At present, the management of fishery wastes is one of the main problems of the greatest concern and impact on the environment [20,21]. In recirculating aquaculture systems, fish sludge and wastes pose a disposal challenge due to their high organic content and moisture, making incineration costly and landfill disposal unsustainable [22]. These effluents, typically high in nutrients, result in algal blooms, offensive odor, acutely lethal

discharges, localized areas of anoxia, etc. [23]. The proper selection of disposal methods and waste processing for the reception of waste is of paramount importance. If large quantities of waste are discharged into a large receiving water body with inadequate mixing and poor water exchange, adverse environmental impact will be observed [24]. To promote sustainable and economical fish farming activities, utilizing the FW generated during farming is essential. Fish processing effluent treatment needs to be considered in order to obtain high-quality water requirements that allow its reuse or recycling for industrial processes [19]. The use of AD to treat fish waste aligns with efforts to promote circular economy practices, reducing environmental pollution while enhancing soil fertility using digestate. Due to its high biodegradability, FW is an ideal co-substrate for AD, providing a complementary nutrient profile that accelerates the breakdown of lignocellulosic materials and improves the overall biogas yield [25].

Phytoremediation is an effective method for treating aquaculture wastewater, enabling its recycling and reuse. Aquatic plant species have been known to drastically reduce the total suspended solids (TSS), ammoniacal nitrogen (NH_3-N), and phosphate from aquaculture wastewater [26,27]. Water hyacinth (*Eichhornia crassipes*) is one of several aquatic macrophytes used in phytoremediation alongside plants such as *Centella asiatica*, *Ipomoea aquatica*, *Salvinia molesta*, and *Pistia stratiotes*. WH exhibits rapid and uncontrollable growth, often completely covering water bodies. It is an excellent accumulator of heavy metals, making it highly suitable for wastewater treatment. The plant primarily absorbs contaminants through its root system [28–31]. WH is a very fast-growing species and has a phenomenal growth rate. The maximum rate of biomass accumulation was estimated to be 20 gm^{-2} day^{-1}, and the maximum relative growth rate was 1.50%/day. The peak efficiency of tissue storage relative to solar input was estimated to be 1.4%. The period of maximum growth occurs in April before the peak standing crop is attained (ca. 2.3–2.5 kg m^{-2}) [32]. WH biomass generated from the phytoremediation system can be effectively used in energy generation, fertilizer production [33], biochar [34], ethanol [35], biogas, and methane [36–40].

The AcoD of lignocellulosic materials with highly biodegradable substrates is an effective strategy for enhancing the biodegradation of lignocelluloses [3]. Various studies have demonstrated that WH exhibits significant biogas potential when co-digested with other substrates [41–44]. WH biomass has a substantial potential for generating energy via biogas and hydrogen [45,46]. Sarto et al. [47] reported that the highest biogas production from WH using cow manure as the inoculum was 424.30 mL, with a maximum methane content of 64.38%. Additionally, a mere 6% increase in gas production with treatment with alkali pretreatment was reported by Patil et al. [48]. The recalcitrant nature of WH may slow down the hydrolysis process and conversion to biogas [49]. The recalcitrant structure of WH can limit microbial degradation and result in slow digestion and reduced biogas yield [50]. The co-digestion of WH biomass with other substrates has been shown to improve the biogas yield. Adding a second substrate seems to improve the methane content and biogas quality [51]. Similarly, the co-digestion of WH with poultry litter produced significantly higher amounts of biogas than pure WH [52]. The combination of acidity and high fat and protein levels makes FW difficult to digest as a sole substrate in AD [53,54]. As a potential solution to the digestion challenges of protein- and lipid-rich FW (energy-dense materials), incorporating a secondary substrate with different properties can help to address the digestion challenges associated with protein- and lipid-rich food waste.

Few studies have investigated the co-digestion of WH and FW for biogas production [55,56]. A notable research gap remains, particularly in enhancing biogas production through the AcoD of WH and FW. Therefore, the objective of the current study was to assess the potential for biogas production from FW and WH, using CD as an inoculum source. As the ratio of inoculum to substrate is a critical factor influencing biogas yield, the current study also examined the effect of ISR on the AcoD of WH and FW.

2. Materials and Methods

2.1. Collection of Substrate and Inoculum

Fish gills and viscera (FW) were collected from the fish shop in Pune, Maharashtra, India (Lat: 18.634052, Long: 73.787463). The wastes were ground in an electric heavy-duty grinder (Hans Dominar X Pro 2200 Watts 3 HP Commercial Mixer Grinder, Model no. RP_001, New Delhi, India) after washing with deionized water to remove dirt, silt, etc. The FW was stored at −20 °C to prevent oxidation and innate fermentation until characterization and use in the experiment. WH was harvested from cultivated tanks that were placed on the rooftop of Defiant Renewables (Lat: 18.633115, Long: 73.790422), Pune, Maharashtra, India. The unrooted WH biomass was blended using a mixer blender and stored at −20 °C prior to AD. The fresh CD was collected from the local cowshed and stored prior to AD. In order to prepare CD slurry, fresh CD was mixed in an equal proportion of water.

2.2. Analytical Methods

TS and VS were determined using a gravimetric analysis by drying the samples at 105 °C for ~2–3 h, and subsequently, they were ashed at 550 °C for 5 h [57]. The total chemical oxygen demand (COD) of the samples was determined using the ASTM D 1252-00 [58] method. The samples were first digested in acidified dichromate using silver sulfate as a catalyst. The digestion was performed by refluxing in a COD digester at 150 °C for 2 h, and the absorbance was determined in a spectrophotometer at 600 nm. The spectrophotometer was calibrated using potassium hydrogen phthalate as a reference standard as per the ASTM D 1252-00. The COD of the digested was monitored every three days. The total Kjeldahl nitrogen (TKN) of the substrate and inoculum was measured using the APHA method [59]. Organic carbon (OC) was measured using the Olsen method [60].

2.3. Measurement of Volatile Fatty Acids and Methane Content

The volatile fatty acids (VFAs) formed in the experiments were measured using an Agilent 7890 B, Santa Clara, CA, USA gas chromatograph fitted with FID and an autosampler. The samples collected from the digestors were centrifuged at 10,000 rpm to remove the solids and extracted with MIBK as an extraction solvent. Methyl-isobutyl-ketone (MIBK) was used as an extraction solvent after painstaking examinations of various solvents and the recoveries of individual VFAs in the selected solvents. The pH of the aqueous sample is lowered using formic acid, which is favored over inorganic acids like phosphoric acid to prevent column damage at the tips. The pH of the sample is lowered to promote the diffusion of VFAs from the aqueous to solvent phase. Sodium sulfate is added to the aqueous sample during the liquid–liquid extraction process. The addition of the salt causes a reduction in the solubility of the VFAs in the raffinate or aqueous phase and increases in the extract or solvent phase. Also, sodium sulfate acts as a drying agent for the extract or solvent phase.

The concentrations of VFAs (acetic acid, propionic acid, iso-butyric acid, n-butyric acid, iso-valeric acid, n-valeric acid, iso-caproic acid, n-caproic acid, and heptanoic acid) were analyzed, and pentanoic acid was used as an internal standard. A DB-FFAP (nitro terephthalic-acid-modified polyethylene glycol (PEG)) column of high polarity for VFAs was used for the analysis. A column of 25 m in length and 0.22 mm diameter with a film thickness of 25 µm was procured from SGE for the same. The analysis method used a nitrogen flow of 3 mL·min^{-1} as a carrier. The oven was programmed from 85 °C for 1 min to 180 °C at a rate of 30 °C min^{-1}. The temperature was finally raised to 220 °C at 40 °C/min. The injector and detector temperatures were set at 230 and 250 °C, respectively.

The biogas generated in the digester was quantified using a gas chromatograph (Agilent 7890 B, Santa Clara, CA, USA), equipped with a TCD and FPD detector. The permanent gases were analyzed using TCD, and sulfur gases were analyzed in FPD. The gas chromatographic conditions used for gas analysis were as follows. A sample loop size of 0.25 mL was used for gas sampling. The nitrogen (N_2) gas (5 mL/min) was used as

the carrier gas for FPD set at 300 °C. Argon (30 mL/min) was used as a carrier gas for TCD maintained at 200 °C with a split ratio of 25:1. Gas sampling was used for sampling analysis. The valves were placed in a valve box maintained at 120 °C. A molecular sieve column (1/8 in diameter and feet long) was used to separate N_2, O_2, H_2, and CH_4. A Porapak column (1/8 in diameter and feet long) was used for CO_2 analysis. A backflush function was provided in the GC to prevent CO_2 poisoning of the molecular sieve column. The oven program was initiated at 45 °C for 6 min, then increased to 180 °C over a period of 2.25 min at a rate of 20 °C/min. The gas generated in the biogas digesters was connected to the GC, and the gas composition was analyzed. The amount of biogas generated from the reactors was calculated using the ideal gas law after accounting for the pressure generated due to water evaporation at STP [61,62].

2.4. Model for Biogas Production and Statistical Analysis

The Gompertz equation was reparametrized in 1990, leading to the development of what is now known as the "Modified Gompertz Equation/Model" [63,64]. This is a sigmoid-shaped growth model characterized by three parameters and is widely applied in biological studies, particularly for modeling the growth of microorganisms and animals. In the context of biogas production, the modified Gompertz equation is used to model kinetic data, assuming that the biogas yield in batch experiments reflects the growth rate of methanogenic bacteria within the biodigester [65,66]. The modified Gompertz model was prepared using Origin Lab Pro (Version 9.9) and calibrated using experimental data.

The statistical analysis of the data was performed using Minitab (version 21.2) software. One-way ANOVA was used to analyze biogas production between different scenarios, and F values were interpreted at a 0.05 significance level. Paired sample *t*-tests were performed to determine the significant difference in biogas production between co-digestion experiments. Fisher's Least Significant Difference (LSD) test was performed to determine the relationship between the biogas production and COD reduction. All the statistical results were analyzed at a 0.05 level of significance.

3. Preparation of Reactors

The reactors were prepared based on the TS and VS content of the inoculum and substrate, with a constant feedstock volume of 400 mL per reactor. The preparation of the reactors followed Equations (1) and (2):

$$\frac{\mathrm{m(IS)} \times \mathrm{VS(i)}}{\mathrm{m(sS)} \times \mathrm{VS(s)}} = \mathrm{Ratio} \quad (1)$$

$$\mathrm{m(sS)} + \mathrm{m\ (IS)} = 400 \quad (2)$$

where m(IS) is the amount of inoculum; m(sS) is the amount of substrate; Vs(i) and VS (s) correspond to the volatile solids of the inoculum and substrate, respectively. The ratios were set at 2 (2:1), 1.5 (3:2), 1 (1:1), and 0.5 (1:2). The volatile solids (%) of the inoculum and WH and FW (substrate) are 5.33, 3.49, and 20.72, respectively.

To optimize the biogas production from WH, FW, and CD, the design of the experiment was split into two experimental setups, as described in Table 1. The first experiment was performed to assess the effect of the WH/FW (substrates) to CD (inoculum) ratios (1:1, 1:2, 2:1, and 3:1) on biogas production (Table 1a). In the second experiment, the optimized co-digestion of FW and WH as substrates in the different ratios (1:1, 1:2, and 2:1) with a fixed amount of inoculum (100 gm) (Table 1b) was undertaken. The experiments are duplicated, with 400 mL of cow dung slurry taken as a blank control.

Table 1. (**a**) Optimization of WH and FW mono-digestion experiments using CD as an inoculum. (**b**) Effect of WH/FW ratio on co-digestion experiments using CD as an inoculum.

(a)				
	Inoculum (g)	**Substrate (g)**	**Ratio**	**Total (g)**
CD:WH (2:1)	226.74	173.25	2	400
CD:FW (2:1)	354.39	45.60	2	400
CD:WH (1:2)	98.61	301.38	0.5	400
CD:FW (1:2)	264.08	135.91	0.5	400
CD:WH (1:1)	158.21	241.78	1	400
CD:FW (1:1)	318.13	81.86	1	400
Blank	400	-	-	400

(b)						
	WH (g)	**FW(g)**	**Ratio**	**Total (g)**	**CD (g)**	**Total (g)**
WH:FW (1:1)	256.76	43.23	1	300	100	400
WH:FW (2:1)	276.70	23.29	2	300	100	400
WH:FW (1:2)	224.41	75.58	0.5	300	100	400

The biogas production experiments were conducted over a period of 24 days in duplicate using reactors constructed from 1000 mL glass bottles, which were hermetically sealed and equipped with outlets for pressure measurement, gas sampling, and liquid sampling. Each reactor had a working volume of 400 mL, with the remaining volume as the headspace. The hermetically sealed bottles were leak tested by pressurizing with nitrogen and monitoring the pressure drop overnight. The nitrogen gas was vented after a successful leak test. Leak-tested bottles were placed in a water bath maintained at 37 °C. They were stirred twice a day. Upon the evolution of gases (N_2, CO_2, and CH_4), the pressure in the bottles rises, as noted by the pressure gauge. The gas generated in the bottles was then analyzed using GC (Agilent 7890 B Gas Chromatograph). The pressure in the bottles was released into the chromatography column through the release valve. The separation of H_2S was carried out using a Gas Pro column and detected by FPD. The GC was also equipped with a back-flush option to prevent poisoning of the molecular sieve. The ideal gas law was used to evaluate the biomethane potential of the substrate. The gas samples were collected within a 6-day interval at the same time while digestate samples were collected within a 3-day interval.

4. Results and Discussion

4.1. Initial Characterization of FW and WH

The initial physicochemical characteristics like the TS, VS, pH, moisture content, ash content, and C/N ratio were analyzed for FW and WH prior to the experimental setup, which is depicted in Table 2. The initial characterization of FW and WH conducted in different studies and the present study is compared and described in Table 2.

4.2. Cumulative Biogas Production and Methane Content

The investigation revealed varying biogas production yields across different inoculum-to-substrate ratios in mono-digestion and co-digestion experiments with a known amount of inoculum. In the case of mono-digestion, the highest biogas production was observed in a 1:2 ratio of CD:FW, i.e., 970 ± 14.14 mL/g VS followed by CD:FW (2:1), CD:FW (1:1), CD:WH (1:1), CD:WH (2:1), and CD:WH (1:2). In the initial experimental phase (day 6), the mono-digestion ratio of CD:FW (2:1) exhibited the highest biogas production, measuring 598.5 ± 20.50 mL/g VS. Conversely, the CD:WH (2:1) ratio demonstrated the lowest biogas production at 167.5 ± 24.74 mL/g VS. Among the co-digestion combinations of WH:FW,

the 1:1 ratio yielded the highest production at 564 ± 73.53 mL/g VS, while the 2:1 ratio resulted in the lowest output of 401.5 ± 23.33 mL/g VS. Between day 6 and day 12, a significant change in biogas production was observed across all reactors (Figure 1).

Table 2. Initial characterization of FW and WH.

Moisture Content (%)	Volatile Solids (%)	Total Solids (%)	Ash Content (%)	pH	C/N Ratio	Reference
FW						
48.6 ± 0.3	96.2 ± 0.5	51.4 ± 0.1	3.8 ± 0.4	7.2 ± 0.001		[67]
74.8	88.9	25.2	-	7.4	5.7	[68]
	28.95	30.63		3.90		[69]
67.1–81.43	27.50–55.5	31.30–32.2	2.14–5.7		3–10.1	[55]
	39.20 ± 0.27	43.54 ± 0.21	2.14 ± 0.05		10.72 ± 0.14	[70]
61.58 ± 2.1	93.84 ± 1.0	38.11 ± 1.9	0.51 ± 0.05	6.42 ± 0.1	5.79 ± 0.12	[56]
	93.74	38.08			8.0	[71]
81.43	-	-	2.14	-	5.01	[72]
68.7	27.50	31.30	5.7	-	4.1	[73]
	20.72	25.93	1.96	6.5	8.69	Present Study
WH						
94.22 ± 2.20	99.48 ± 1.11	5.58 ± 0.06	16.65 ± 0.35	7.19 ± 0.12	21.25 ± 0.25	[56]
95.2	67.61	-	-	5.82	25.7 ± 1.3	[37]
-	87.4 ± 1.1	12.4 ± 1.3	-	-	28.8 ± 1.5	[74]
-	86.2 ± 0.9	11.4 ± 1.4	-	-	29.0 ± 1.3	[40]
86.5 ± 1.52	78.95 ± 1.44	102.56 ± 4.32	4.52 ± 0.07	-	18.12 ± 0.23	[75]
-	3.49	4.09	0.121	6.98	22.93	Present Study

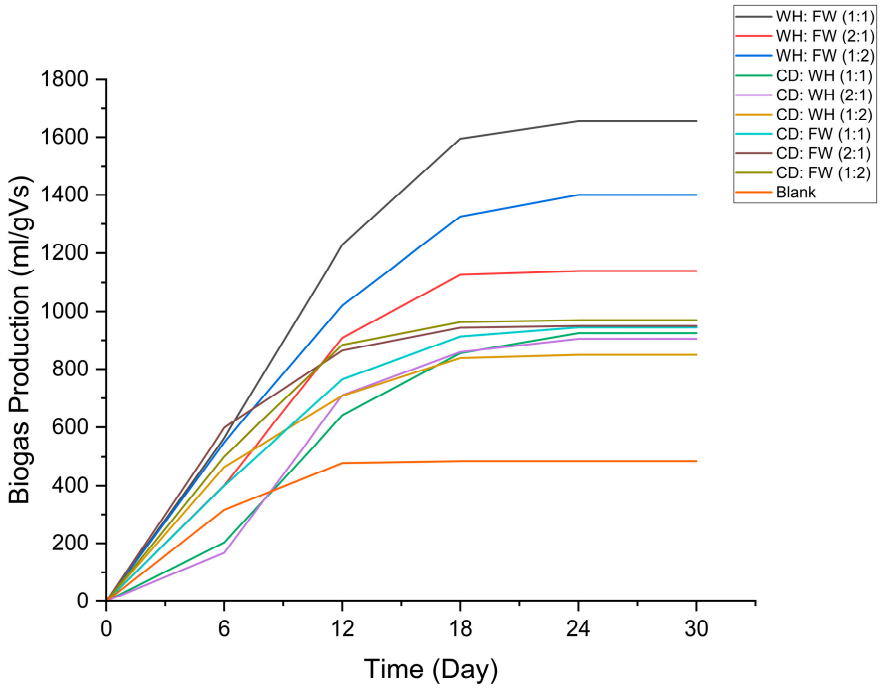


Figure 1. Cumulative biogas production from mono-digestion and co-digestion.

In the case of the co-digestion of WH and FW with fixed inoculum (CD), the highest biogas production was observed to be 1655 ± 91.92 mL/g VS in a 1:1 (WH:FW) ratio and 1400 ± 56.56 mL/g VS in 1:2 (WH:FW) ratio. The co-digestion ratio of 2:1 (WH:FW) also exhibited a considerable biogas production of 1140 ± 169.70 mL/g VS, which is also greater than mono-digestion (Figure 2a). The biogas production increased by approximately 70.62% when moving from the highest yield in mono-digestion to the highest yield in co-digestion. The observed increase in biogas production during co-digestion compared to mono-digestion can be attributed to several synergistic factors that enhance the overall anaerobic digestion process. Co-digestion, involving the combination of substrates such as WH and FW, results in a more balanced nutrient profile, particularly in terms of the C/N ratio which is crucial for optimal microbial activity.

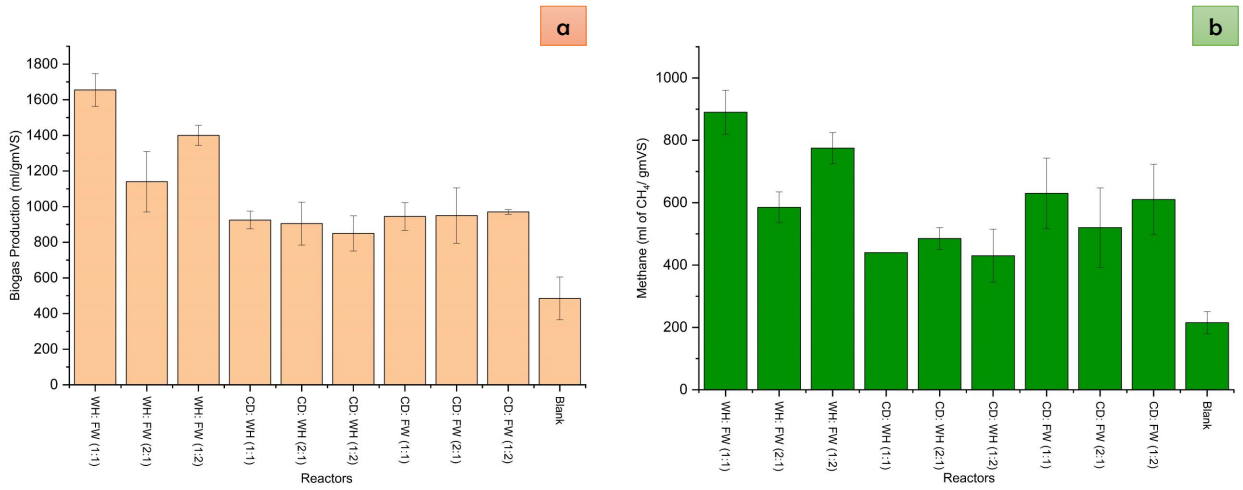


Figure 2. Cumulative average production ((**a**) biogas production and (**b**) methane yield).

In mono-digestion, the highest methane yield was observed in the CD and FW at a 1:1 ratio, measuring 630 ± 113.13 mL CH_4/g VS, followed by CD:FW (1:2), CD:FW (2:1), CD:WH (2:1), CD:WH (1:1), and CD:WH (1:2), respectively. During the co-digestion, the average methane production exhibited higher values in the 1:1 and 1:2 mixtures of WH and FW, i.e., 890 ± 70.71 mL CH_4/g VS and 775 ± 49.49 mL CH_4/g VS, respectively, followed by the 2:1 ratio (585 ± 49.49 mL CH_4/g VS) (Figure 2b). In mono-digestion, the cumulative biogas and methane production collectively indicated that FW yielded a higher biogas production than WH. Moreover, the co-digestion of two substrates (WH and FW) notably enhanced gas and methane production compared to mono-digestion. FW might be more readily digestible, providing an immediate source of easily degradable organic matter.

Analyses of the variation in the production of biogas between co-digestion, mono-digestion, and overall (both co-digestion and mono-digestion) digestion are presented in Supplementary Table S1. Analysis showed that in the case of co-digestion and mono-digestion, the models were non-significant ($p < 0.310$ and $p < 0.824$, respectively), whereas the overall biogas production from various substrates showed a significant result ($p < 0.024$). It may be concluded that production efficiency greatly varies among the different varieties of substrates, and future studies should focus on the specific substrates and different ratios.

Paired sample t-tests between different co-digestions are presented in Supplementary Table S2. The table shows that the differences between WH:FW (1:1) and WH:FW (2:1) and WH:FW (2:1) and WH:FW (1:2) were highly significant ($p < 0.004$; $p < 0.003$, respectively). The difference between WH:FW (1:1) and WH:FW (1:2) also showed significant results but less significant than the other two co-digestions. In the comparison of biogas production during the co-digestion experiments, the ANOVA values did not indicate significant variations among them. On the other hand, the ANOVA results for mono-digestion revealed significant variations in biogas production.

Hortence et al. [56] conducted a similar investigation on biogas production utilizing FW and WH, where the highest methane content of 68.15% was observed in a substrate ratio of 25:75 g (WH:FW). Nalinga and Legonda [76] reported the highest biogas production from the mixture of WH and FW in a ratio of 1:2, producing biogas of 0.55 L, with a corresponding methane content of 73.3%. Nazurally [67] explored the biogas production potential of FW and seagrass combined with macroalgae using 2000 mL of plastic bottles, achieving a maximum cumulative biogas production of 8410 mL with a mixture of 60% FW and 40% seagrass/microalgae. Mshandete et al. [77] investigated the anaerobic batch co-digestion of sisal pulp and FW, identifying the highest methane yield of 0.62 CH_4 m^3/kg VS (i.e., 620 CH_4 L/kg VS) when FW and sisal pulp were mixed in a ratio of 33:67. Cadavid-Rodríguez et al. [68] examined anaerobic digestion with various concentrations of TS in FW and obtained the maximum biochemical methane potential (BMP) of 464.55 mL CH_4/g VS from 1% TS. Sarker [69] conducted anaerobic digestion of FW with different substrates from a fish-oil refinery, achieving a maximum biogas production of 2089 mL from a mixture of FW and light ethyl monoester, with a methane production of 72%. Xu et al. [71] investigated anaerobic digestion using FW, bagasse collected from a sugar mill as a substrate, and municipal sludge as an inoculum, observed a maximum biogas yield of 409.5 mL/g VS with a 73.34% methane content from the mono-digestion of FW, compared to co-digestion with bagasse producing biogas with a 67.8% methane content. Kafle and Kim [73] reported a biogas yield of 757 mL/g VS from FW after 60 days of digestion, with a methane yield of 554 mL/g VS^{-1} and a methane content of 73%. Bücker et al. [78] investigated anaerobic digestion using FW and fish crude oil waste, obtaining methane contents of 540.5 CH_4 mL/g VS and 426.36 mL/g VS, respectively, using anaerobic microbiota from a biogas plant sludge mixture as the inoculum. It can be noted that the highest biogas yield was observed in the present study when using WH and FW in co-digestion and mono-digestion experiments, when compared to other studies. Different studies conducted by various researchers on mono-digestion and co-digestion using FW and WH as substrates with different inoculum are summarized in Table 3. The results from these studies indicate that biogas production from mono-digestion is lower compared to co-digestion, which aligns with the present study's findings. Our results confirm this trend, showing a significant increase in biogas production when WH and FW are co-digested, underscoring the advantages of substrate synergy in anaerobic digestion.

Table 3. Biogas and methane production in different studies and the recent study.

Inoculum	Substrate	Operating Condition	Biogas Yield/BMP (mL/g VS^{-1})	Methane (%)	Reference
Sludge	FW, seagrass, microalgae	ISR: 1:3 HTR: 26 days	8410 *[a]		[67]
CD	FW	ISR: 1:1.2 HTR: 15 days Temp: Ambient	2 *[b]		[79]
Industrial inoculum	FW	HTR: 20 days	1546 *[a]	17.7	[80]
Anaerobic sludge from wastewater treatment reactor	FW	HTR: 28 days Temp: Mesophilic	464.5	75.5 *[c]	[68]
Anaerobic digestion sludge	Ensilaged FW Soap stock, alkaline fish glycerin, light ethyl monoester, dark ethyl monoester	HTR: 65 Days Temp: 39 ± 1	FE: 985 *[a] FE + SS: 1738 *[a] FE + AFG: 1818 *[a] FE + LME: 2089 *[a] FE + DME: 1948 *[a]	FE: 73 FE + SS: 69 FE + AFG: 72 FE + LME: 72 FE + DME: 71	[69]
	Fish intestines			50.12	[55]

Table 3. *Cont.*

Inoculum	Substrate	Operating Condition	Biogas Yield/BMP (mL/g VS^{-1})	Methane (%)	Reference
Sludge from the anaerobic fermentation tank	Fish processing waste, bamboo hydrochar	ISR: 1:2 HRT: 36 days Temp: 37 ± 1 °C	292 *[b]	74.9	[70]
Digestate from biogas plant	FW, WH	ISR: 25:75 HRT: 20 days Temp: 37 °C	700 *[a] (approx.)	68.15	[56]
Sludge of municipal anaerobic digester	FW	HRT: 21 days Temp: 37 ± 1 °C	433.4	73.34	[71]
Sludge of municipal anaerobic digester	FW, bagasse	ISR: 2.19 HRT: 21 days Temp: 37 ± 1 °C	409.5	67.8	[71]
Swine manure	FW, bread waste silage	ISR: 80:20 HRT: 96 days	763	63.1	[81]
Swine manure	FW, brewery grain waste	ISR: 40:60 HRT: 96 days	671	65.8	[81]
Swine Manure	FW	HRT: 60 days Temp: 36.5 °C	757	73	[73]
CD and blood with a ratio of 1:1	FW WH	ISR: 1:1, 1:2 and 2:1 Temp: 25.3 °C to 33.4 HRT: 21 days	FW: WH (1:1)–0.30 *[d] FW: WH (1:2)–0.55 *[d] FW: WH (2:1)–0.35–0.45 *[d] (Approx.)	FW: WH (1:1)–63 FW: WH (1:2)–73.3 FW: WH (2:1)–65	[76]
Anaerobic microbiota of a biogas plant sludge mixture	FW	HRT: 20 days Temp: 35 °C	791.78	540.5 *[c]	[78]
Anaerobic microbiota of a biogas plant sludge mixture	Fish crude oil waste	HRT: 20 days Temp: 35 °C	733.93	426.36 *[c]	[78]
WH					
Cow manure and sewage sludge	WH	ISR: HRT: Temp: 37 °C	812 *[a]	65	[82]
CD	WH (pretreated 5% NaoH)	ISR: 2:1 HRT: 25 days Temp: 37 °C	142.61 *[b]	64.59	[37]
Cow manure	WH	ISR: 2:1 HRT: 30 days Temp: 37 °C	410 *[b]	-	[38]
Waste activated sludge	WH	F/M: 1.5 HRT: 35 days Temp: 35 °C	2053 *[a]	54.67 ± 0.6	[75]

Table 3. *Cont.*

Inoculum	Substrate	Operating Condition	Biogas Yield/BMP (mL/g VS^{-1})	Methane (%)	Reference
Present Study (Co-Digestion)					
CD	WH FW	WH:FW: 1:1 HRT: 24 days Temp: 37 °C	1655 ± 91.92	890 ± 70.71 *c	Present Study
CD	WH FW	WH:FW: 1:2 HRT: 24 days Temp: 37 °C	1400 ± 56.56	775 ± 49.49 *c	Present Study
CD	WH FW	WH:FW: 2:1 HRT: 24 days Temp: 37 °C	1140 ±169.70	585 ± 49.4 *c	Present Study

Where *a mL, *b Lit kg^{-1} VS^{-1}, *c CH_4 mL/g VS, *d Lit.

4.3. Modified Gompertz Model

The modified Gompertz model was fitted to the experimental data to predict biogas production under various conditions like mono-digestion and co-digestion with different inoculum-to-substrate ratios. Figure 3 shows a comparison of the predicted and experimental cumulative biogas production. The actual biogas (Y) production values from the co-digestion of WH and FW were 1655, 1140, and 1400 mL/g VS for ratios of 1:1, 2:1, and 1:2, respectively. Under mono-digestion conditions of CD and WH, the maximum biogas yields were 925, 905, and 850 mL/g VS for the 1:1, 2:1, and 1:2 ratios, respectively. Furthermore, in the case of the mono-digestion of CD and FW, the biogas yields were 945, 950, and 970 mL/g VS for the 1:1, 2:1, and 1:2 ratios, respectively. The fitted model yielded maximum biogas yields (Ym) of 1683, 1154, and 1422 mL/g VS for WH and FW co-digestion at 1:1, 2:1, and 1:2 ratios, respectively. Similarly, in the case of CD and WH mono-digestion, the predicted maximum biogas yields were 937, 903, and 846 mL/g VS for the 1:1, 2:1, and 1:2 ratios. In the CD and FW mono-digestion experiments, the corresponding Ym values were 945, 950, and 970 mL/g VS for the 1:1, 2:1, and 1:2 ratios.

The ultimate biogas yield (asymptotic values) denoted by K were determined as 0.223, 0.259, and 0.205 for the 1:1, 2:1, and 1:2 ratios, respectively, in the WH and FW co-digestion. In the CD and WH mono-digestion, the corresponding K values were found to be 0.234, 0.320, and 0273 for the ratios 1:1, 2:1, and 1:2. In the CD and FW WH mono-digestion studies, the measured K values were 0.248, 0.355, and 0.334 for the 1:1, 2:1, and 1:2 ratios, respectively. The coefficient of determination (R^2) was determined to be 0.998, 0.998, 0.995, 0.999, 0.999, 0.990, 0.998, 0.996, and 0.999 for all samples. Notably, all R^2 values approached unity (1), indicating a high degree of conformity between the experimental findings and simulated values. Moreover, it signifies the precision and dependability in the correlation between experimental observations and model predictions.

The CD and FW mono-digestion studies exhibited a lag phase of approximately 1 day across three different ratios. In the case of WH and FW, co-digestion in a 1:2 ratio also exhibited a lag phase of 1 day. In contrast, the CD and WH mono-digestion mixture displayed comparatively longer lag phases, specifically 3.55, 4.49, and 3.75 days for the 1:1, 2:1, and 1:2 ratios, respectively. The WH and FW co-digestion at 1:1 and 2:1 demonstrated moderate lag phases of 2.02 and 2.39 days, accompanied by the highest biogas production. It can be observed that from the model prediction, the higher amount of FW plays a crucial role in reducing the lag phase and enhancing the biogas production (Supplementary Table S3).

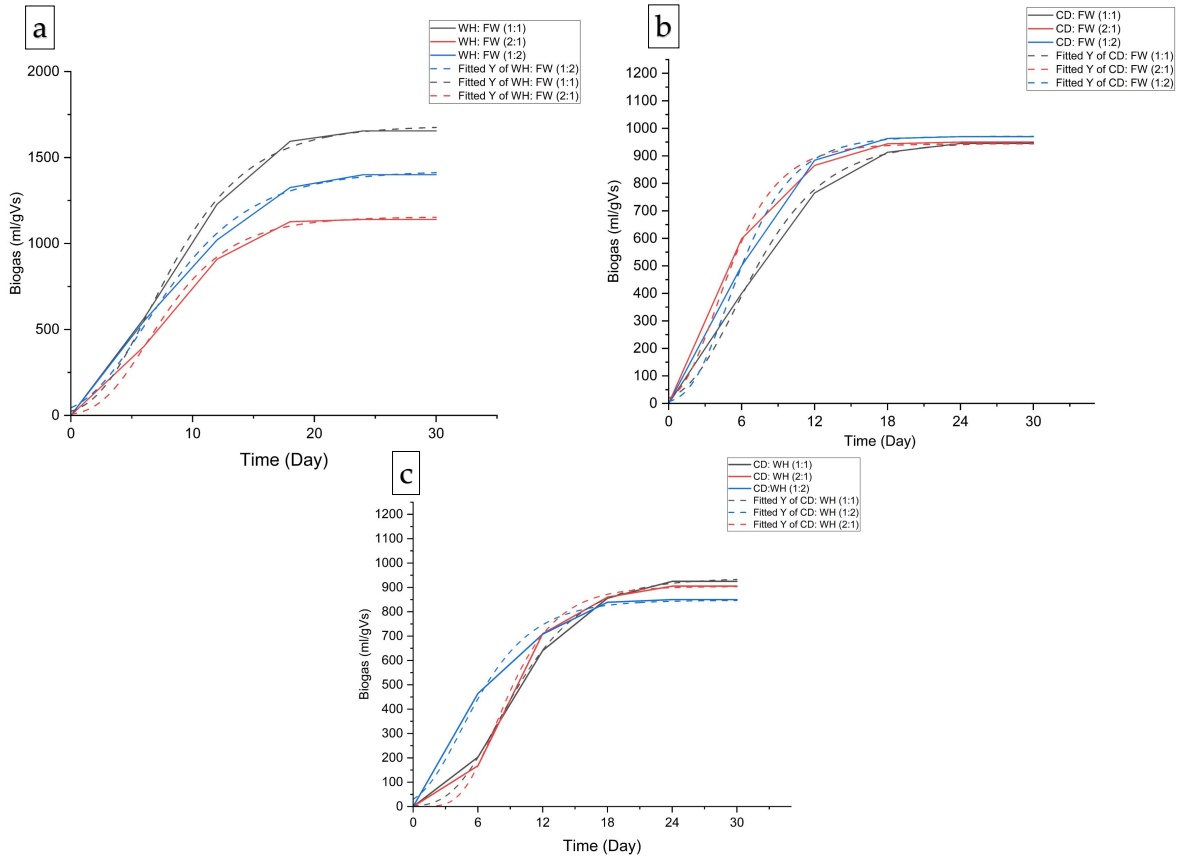


Figure 3. Modified Gompertz model (**a**): Co-digestion of WH and FW; (**b**): Mono-digestion of FW with CD; (**c**): Mono-digestion of WH with CD.

4.4. Removal of Chemical Oxygen Demand

A reduction in chemical oxygen demand (COD) was observed during the anaerobic digestion process. On day 3, the highest COD level was recorded for the CD:FW (2:1) ratio, measuring 8001 ± 776.40 mg/L, while the blank control exhibited the lowest COD at 4181 ± 1132.78 mg/L. Among the co-digestion ratios, WH:FW (1:2) showed the highest COD at 7470 ± 212.13 mg/L, followed closely by WH:FW (2:1) at 7429 ± 154.14 mg/L and WH:FW (1:1) at 6433.5 ± 48.79 mg/L (Figure 4).

The highest COD reduction percentages were achieved in the blank sample, registering a 94.48% reduction, suggesting inherent processes or factors contributing to COD reduction in the absence of specific treatment components. Following closely were the mono-digestions involving CD with WH, notably the CD:WH (1:1) and CD:WH (2:1) combinations, which demonstrated robust COD reduction percentages of 91.68% and 90.99%, respectively. Moreover, combinations of CD with FW in mono-digestion also exhibited notable efficacy, ranging from 86.45% to 90.98%. However, slightly lower COD reduction percentages were observed in the combinations involving equal proportions of CD and FW (1:1). Figure 4 shows the removal of COD during the AD experiments.

An analysis of the variation in the reduction in COD during overall (both co-digestion and mono-digestion) digestion is presented in Supplementary Table S4. Analysis showed reduction in COD from various substrates showed a significant result ($p < 0.001$), which implied a graduation reduction in COD while graduation increase in biogas production.

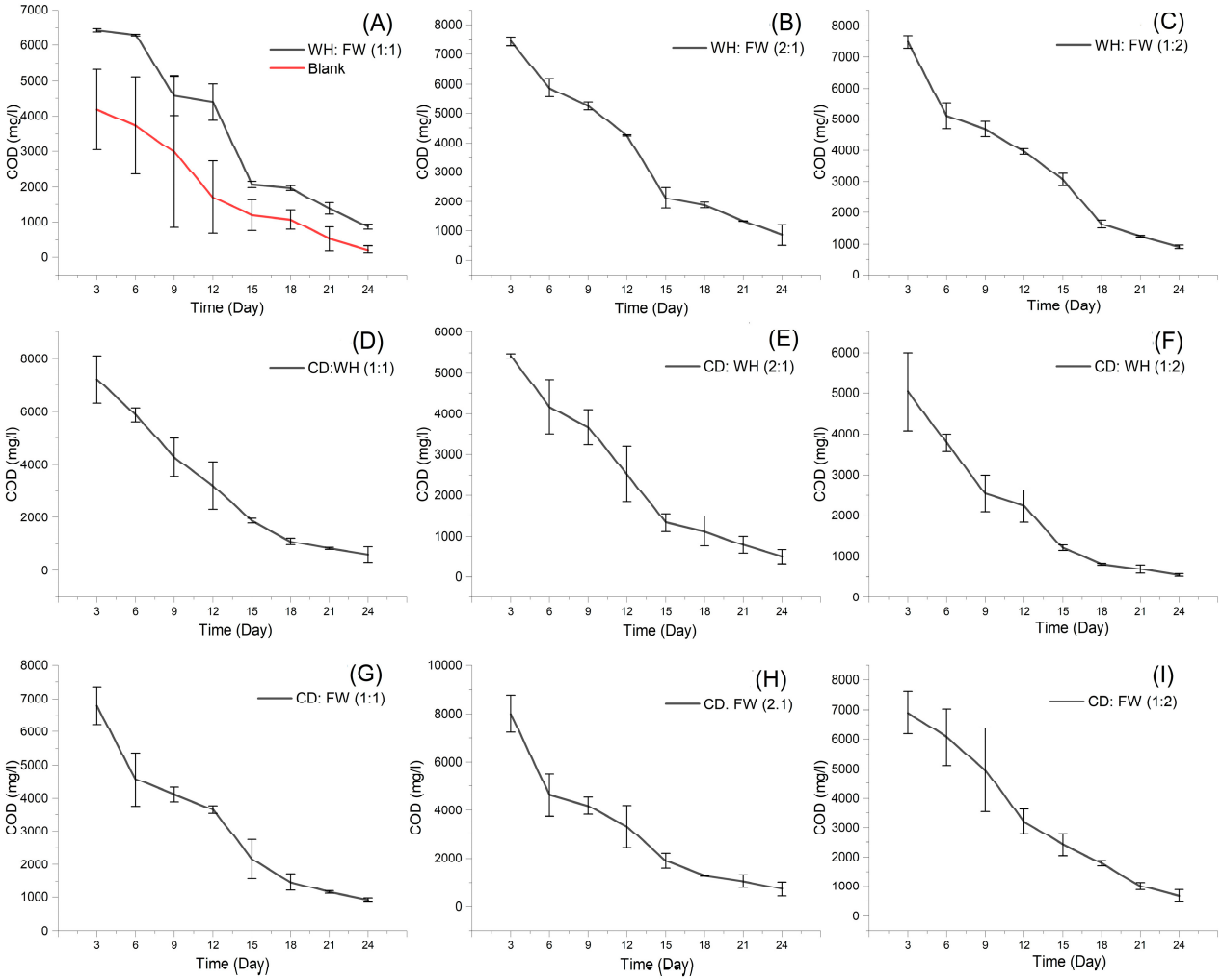


Figure 4. Reduction in COD during anaerobic digestion. (**A**) WH:FW (1:1); (**B**) WH:FW (2:1); (**C**) WH:FW (1:2); (**D**) CD:WH (1:1); (**E**) CD:WH (2:1); (**F**) CD:WH (1:2); (**G**) CD:FW (1:1); (**H**) CD:FW (2:1); and (**I**) CD:FW (1:2).

In a study conducted by Choi [83], anaerobic co-digestion experiments were carried out utilizing fish by-products over 30 days at a temperature of 35 °C. The investigation assesses the reduction in COD in samples with fishery by-products broth. The results indicated COD reductions of 45.32%, 51.26%, and 46.66% for the respective mixtures of 70% sludge and 30% by-products broth, 50% sludge and 50% by-products broth, and 30% sludge and 70% by-products broth. The highest reduction was observed when the experiments were performed using 50% sludge and 50% by-product broth.

4.5. Biogas Production and COD Reduction

COD is crucial for maximizing biogas yield and ensuring the efficient operation of the anaerobic digestion process. COD is a vital parameter in biogas production, as it quantifies the organic material available for conversion into biogas within an anaerobic digester [84]. The reduction in COD during anaerobic digestion is attributed to the breakdown of organic matter into gaseous products, such as methane and carbon dioxide, from the feedstock slurries [85]. In the present study, the CD:FW (2:1) ratio demonstrated the highest COD value of 8001 $\pm$ 776.40 mg/L on day 3, as well as the highest biogas production, measuring 598.5 $\pm$ 20.50 mL/g VS on day 6. This correlation indicates the significant contribution of COD to biogas production.

In the present study, the overall mean values of biogas production and overall COD reduction were observed by Fisher's Least Significant Difference test, which is represented in Table 4. Fisher's LSD method compares the means of each factor, and means that do not share a letter are significantly different. From the table, it can be observed that in co-digestion, the production of biogas from WH:FW (1:1) and WH:FW (2:1) showed a significant difference from other digestions, including WH:FW (2:1). It was also noted that during mono-digestion, the biogas production values from CD:WH (1:2), CD:WH (1:1), and CD:WH (2:1) are completely different from other digestions, and the differences were significant. On the other hand, the mean COD reduction values for days 3, 6, 9, 12, and 15 were significantly different except for on days 18 and 21.

Table 4. Fisher's Least Significant Difference test of biogas production and COD reduction.

	Reactors	Mean	Grouping				COD	Mean	Grouping	
Biogas production	WH:FW (1:1)	1339.3	A			Reduction in COD	COD 3	6488.6	A	
	WH:FW (1:2)	1139.0	A	B			COD 6	5013.4	B	
	WH:FW (2:1)	943.4		B	C		COD 9	4123.8	C	
	CD:FW (2:1)	861.5		B	C		COD 12	3248.5	D	
	CD:FW (1:2)	857.5		B	C		COD 15	1936.3	E	
	CD:FW (1:1)	793.6		B	C		COD 18	1414.0	E	F
	CD:WH (1:2)	742.3			C		COD 21	1001.4		F
	CD:WH (1:1)	709.8			C					
	CD:WH (2:1)	709.5			C					

4.6. Reduction in Volatile Fatty Acids

Figure 5 shows the VFA concentrations for both co-digestion and mono-digestion of WH, FW, and CD in different ratios. The highest VFA concentrations were consistently observed in certain mixtures. The WH:FW (1:2) co-digestion had the highest concentrations for most VFAs, with acetic acid at 1069.97 mg/L, followed by propionic acid at 644.63 mg/L and butyric acid at 170.95 mg/L. The CD:FW (1:2) mono-digestion also showed elevated levels, particularly of acetic acid (986.09 mg/L) and butyric acid (135.80 mg/L). Lower concentrations were generally found in CD:WH combinations for all VFAs, while the blank sample consistently had the lowest VFA levels, serving as a reference for comparison. The higher VFA production observed in samples containing FW can be attributed to its rich and easily degradable organic content. FW is composed of significant amounts of carbohydrates, proteins, and lipids that are quickly broken down by anaerobic microorganisms during digestion, forming VFAs such as acetic acid, propionic acid, and butyric acid. Carbohydrates in FW are rapidly converted into simple sugars and subsequently fermented into VFAs by acidogenic bacteria.

Additionally, proteins and lipids in FW contribute further to VFA production through their breakdown into amino and long-chain fatty acids, respectively. This rapid degradation of FW contrasts with substrates like WH, which contains more fibrous material that requires longer digestion time, resulting in lower VFA production. Consequently, the high VFA concentrations observed in FW samples underscore the substrate's suitability for co-digestion, enhancing biogas production due to its readily fermentable organic matter.

Elevated levels of VFA were prominently noted on days 6, 9, 12, and 15, coinciding with periods of notably lower biogas production which increased steadily. Diminished VFA concentrations were observed on days 18, 21, and 24, registering notably reduced VFA levels, suggesting a temporal decline in VFA concentrations over the course of the observation period. The reduction in volatile fatty acids along with the biogas production is described in Figure 6.

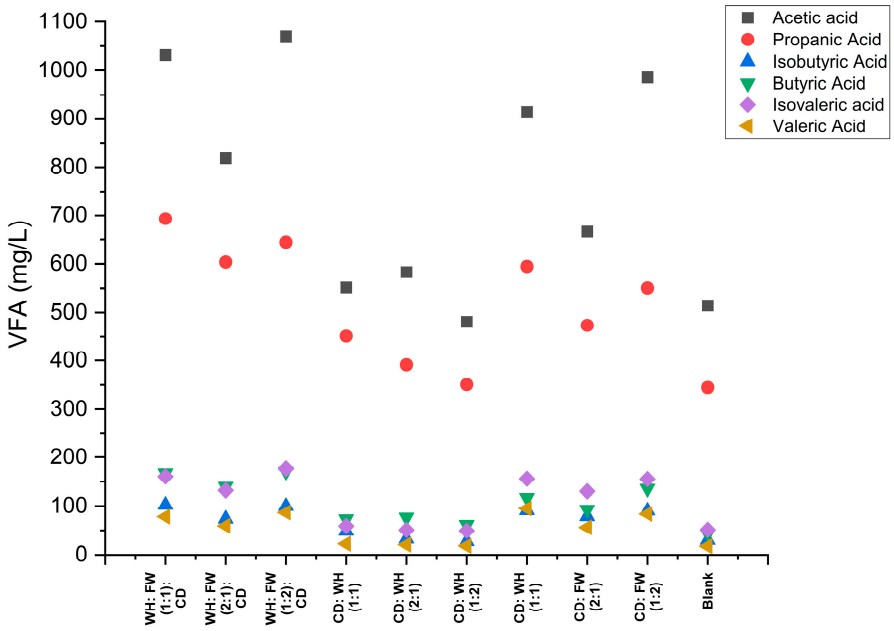


Figure 5. Total volatile fatty acids generated in mono- and co-digestion.

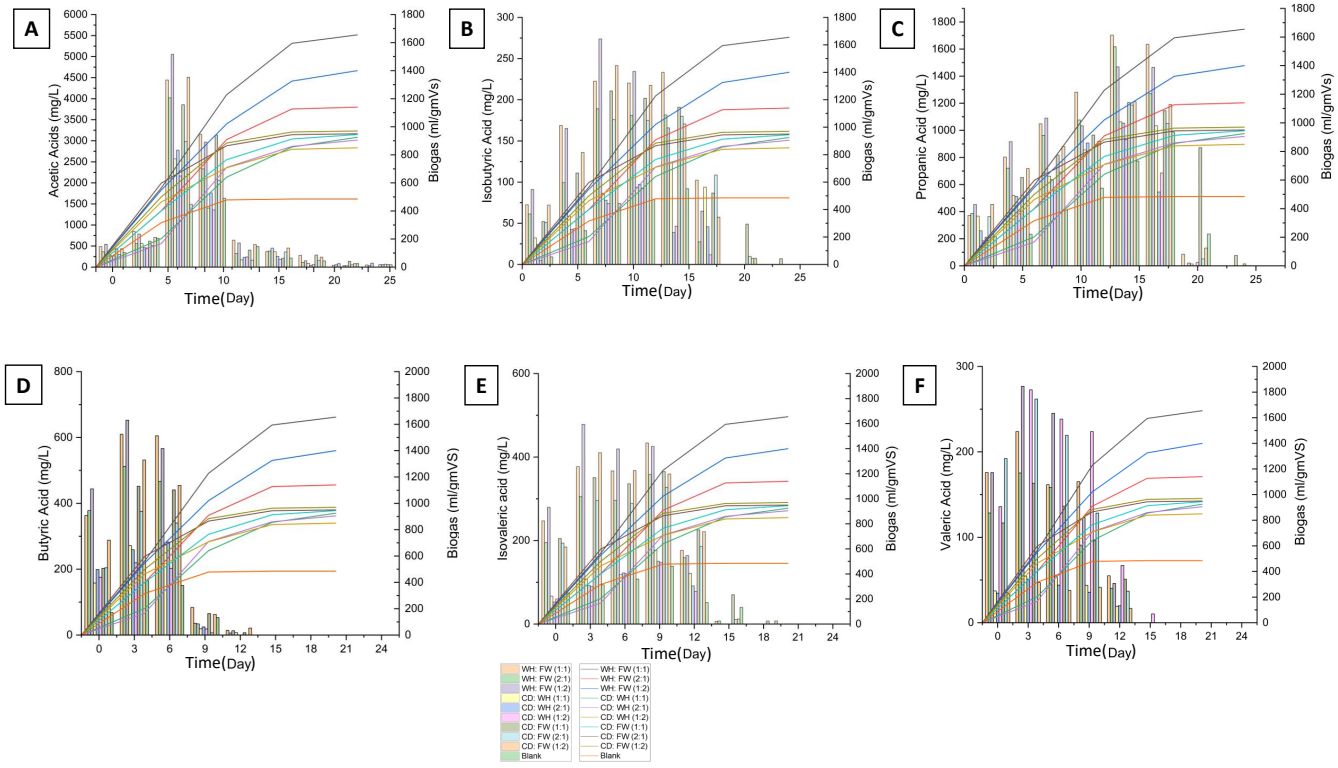


Figure 6. Changes in volatile fatty acids and biogas production with respect to time. (**A**) Acetic acid and biogas production; (**B**) isobutyric acid and biogas production; (**C**) propanic acid and biogas production; (**D**) butyric acid and biogas production; (**E**) isovaleric acid and biogas production; (**F**) valeric acid and biogas production.

4.7. Propionic Acid and Acetic Acid Ratio

The propionic and acetic acid ratio suggests that values above 1.4 may signify a potential inhibition of methane production [74,86]. Interestingly, none of the ratios on any of the observed days exceeded this threshold. The highest ratios were recorded on day 12, with values ranging from 2.26 to 7.16 across various sample compositions. However, despite

these elevated ratios, none crossed the 1.4 threshold. This suggests that the propionic to acetic acid ratio did not indicate a clear inhibition of methane production, as the values stayed below the critical threshold. Although the ratios increased significantly on day 12, they remained within safe limits, implying that the VFA levels likely did not hinder methane production during the observed period for the sample compositions (Table 5).

Table 5. Ratio of acetic acid and propionic acid.

Time (Day)	WH:FW (1:1): CD	WH:FW (2:1): CD	WH:FW (1:2): CD	CD:WH (1:1)	CD:WH (2:1)	CD:WH (1:2)	CD:FW (1:1)	CD:FW (2:1)	CD:FW (1:2)	Blank
0	0.76	1.06	0.83	1.02	0.95	0.70	0.49	1.21	1.04	0.63
3	0.95	1.30	1.17	0.93	1.11	0.89	1.06	0.98	1.02	0.34
6	0.24	0.24	0.22	0.26	0.23	0.26	0.21	0.23	0.20	0.28
9	0.41	0.46	0.35	0.61	0.52	0.53	0.31	0.43	0.32	0.35
12	2.67	5.00	2.56	6.19	4.56	3.87	2.99	7.16	2.26	1.58
15	4.43	3.32	3.26	2.84	2.14	3.78	5.49	2.91	2.64	4.62
18	0.31	0.00	0.14	0.16	0.00	0.41	3.04	0.32	0.56	1.58
21	0.00	0.00	0.00	0.00	0.00	0.00	0.56	0.00	0.00	0.17
24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

5. Conclusions

The study demonstrated that FW and WH are promising feedstocks for biogas production using cow dung as an inoculum. Co-digestion in small reactors under controlled temperature conditions showed a higher biogas production potential as compared to mono-digestion. The current research showed that WH:FW (1:1) and WH:FW (1:2) could be an important co-digestion pair for significant biogas production. This research also showed that COD varied significantly among all reactors, which suggested that it is a good predictive variable for biogas production assessment. The fitted Gompertz model indicates a high degree of conformity between the experimental findings and simulated values for both mono- and co-digestion. The model also predicted that the reduction in lag phase depends critically on increasing the quantity of FW. The current study was conducted on a small scale with reactors using limited factors to access biogas production. Further study on the co-digestion of FW and WH needs to be carried out in larger reactors to determine the operational condition, scale effect, and economic criteria to prove its applicability for large-scale implementation.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app14219880/s1, Table S1: Variation of production of biogas between Co-digestion, mono-digestion and overall (both Co-digestion and mono-digestion) digestion; Table S2: Pair sample *t*-test of biogas production between different co-digestions; Table S3: The kinetics parameters of the fitted model; Table S4: Analysis of overall variation of reduction of Chemical Oxygen Demand.

Author Contributions: Conceptualization, methodology, formal analysis, visualization, writing—original draft, G.N. and A.K.; software, validation, visualization, A.K. and S.G.; project administration, funding acquisition, writing—review and editing, investigation, supervision, A.B.R. and S.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Gaurav Nahar was employed by the company Defiant Renewables Pvt Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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