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Elucidating Synergetic Effects of Anaerobic Co-Digestion of Slaughterhouse Waste with Livestock Manures

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Abstract: This study quantitatively analyzed the synergistic effects of co-digestion of slaughterhouse waste (SHW) with cattle manure (CM) and pig manure (PM) on methane production by applying statistical methods. The biochemical methane potential of volatile solid concentration-based mixtures showed that the biodegradability (BD) of the co-substrates was improved as the mixing proportion of the highly biodegradable SHW increased. Furthermore, mathematical analysis using the modified Gompertz model showed that an increase in the SHW mixture ratio shortened the lag phase at the initial period by more than 58%. The synergy index (SI) analysis revealed that co-digestion of CM and SHW mixed at an equal ratio of 1:1 in sample S4 resulted in a higher SI of 1.18 compared to 1.10 for PM and SHW in sample S5. An overlay plot based on BD and SI identified the optimal mixture ratio as 26.9:31.0:42.1 (CM/PM/SHW), where both BD and SI reached their maximum values. The study successfully demonstrated that co-digestion of SHW with livestock manure enhances BD through a synergistic effect.

Keywords: anaerobic co-digestion; slaughterhouse waste; livestock manures; mixture design

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1. Introduction

Rapid population growth and rising human living standards in South Korea have led to a significant increase in meat consumption. Per capita consumption has doubled from 31.9 kg in 2000 to 62.5 kg in 2022. Notably, South Korea ranks first among the organization of economic cooperation development (OECD) countries in terms of annual pork consumption. This surge in meat consumption has driven substantial growth in South Korea's meat processing industry, making it one of the top ten pork-producing nations [1]. A notable portion of an animal's weight ranging from 20 to 50%, consists of parts unsuitable for human consumption, resulting in the generation of slaughterhouse waste (SHW) [2]. These waste materials, including blood, feathers, viscera, fat, and skin, contain significant amounts of protein and lipids. They are typically processed in rendering plants to produce nutrient-rich animal feed. However, the rendering process requires substantial energy for activities like centrifuging, cooking, and drying. In some cases, it requires electricity of over 70 kWh/ton SHW and process fuel of over 639 kWh/ton SHW, thereby diminishing the economic value [3]. Moreover, in South Korea, the primary disposal methods for SHW are incineration and burial. These approaches either require a significant amount of energy and produce air pollutants or necessitate a large land area for implementation. Given its high organic content, SHW poses challenges for conventional treatment, necessitating careful planning to manage animal-derived diseases, safeguard public health, and minimize its environmental impact [4].

Anaerobic digestion (AD) has emerged as a promising technology for treating SHW due to its potential for biomethane production and minimal environmental impact. The high protein and lipid content of SHW has significant potential for biomethane production [5,6]. The biochemical conversion of SHW into biogas involves several stages. Initially, the SHW undergoes hydrolysis, forming amino acids and long-chain fatty acids. Subsequently, in the acidogenesis and acetogenesis process, these compounds are transformed into ammonia, hydrogen sulfide, volatile fatty acids, hydrogen, and carbon dioxide. Finally, methanogens facilitate the production of biogas during the methanogenesis stage. However, this process is delicate and susceptible to failure. The accumulation of long-chain fatty acids presents a critical bottleneck in the hydrolysis process and can lead to toxicity issues in acetogenic bacteria and methanogenic archaea [7,8]. Additionally, the residues often exhibit low alkalinity and form floating scum during AD [9]. Ammonia in its non-ionized form is known to inhibit the process, with concentrations ranging from 0.1 to 1.1 kg/m^3 proving to be inhibitory [10]. Due to the elevated nitrogen and total solids content of SHW, it is uncommon for it to be treated in its original undiluted state.

In this regard, an attractive approach is the co-digestion of SHW with other low-concentration organic wastes, such as manure or wastewater. Incorporating dilute streams not only improves the overall process stability but also serves as a dilution medium for effectively treating the residual stream [11]. Different combinations of wastes are suitable for co-digestion with SHW. Co-digestion with the organic fraction of municipal solid waste, sewage sludge, fruit and vegetable waste, and other industrial wastes such as saw dust have also been studied [12]. However, the success of the co-digestion strategy for SHW depends on the selection of co-substrates with complementary characteristics. Livestock manure, such as cow manure (CM) and pig manure (PM), offers significant advantages such as balanced macro- and micronutrient contents, microbial metabolism, buffer capacity, biodegradability, and the dilution of toxic compounds [13]. CM is rich in alkali and nutrients, making it suitable for co-digestion with rapidly degradable carbohydrate-rich substrates [14]. PM has a higher buffer capacity and a diverse range of micro- and macronutrients that are essential for the growth and activity of anaerobic microorganisms [15]. However, in co-digestion processes involving two to three substrates, the composition of the mixture significantly affects the biogas yield, process stability, and solids degradation rate. The traditional method for determining the optimal conditions requires a large number of experiments and time, resulting in less accurate results.

The mixture experimental design is a valuable technique for assessing the interaction among the components of a blend to maximize the response. Mixture design using design of experiments (DOE) allows experiments to be designed on the basis of the number of selected factors, enabling an analysis of the interactions between these factors [16]. The results obtained through this approach are more accurate and precise compared to traditional methods. By selecting different factors and determining their levels, the optimal conditions can be derived based on understanding their relationships and the response obtained during the experiments [17]. Design expert (Stat-Ease, Inc., Minneapolis, MN, USA) is a widely used tool for designing experiments and can yield effective results while conducting a minimal number of experiments. Therefore, this study aimed to quantitatively elucidate the synergetic effects of co-digesting ternary mixtures of SHW with CM and PM on methane production by applying the response surface methodology and mixture design tools supported by the Design Expert (v13) software .

2. Materials and Methods

2.1. Inoculum and Substrates

An inoculum was obtained from a full-scale AD reactor operating at a wastewater treatment facility in Gwangju, South Korea. The organic materials (CM and PM) used as substrates were directly collected from local livestock farms, whereas the SHW was collected from a pig slaughterhouse facility in Gwangju, South Korea. Upon collection, the CM and PM were sieved for uniformity and stored in a freezer at −20 °C. The SHW comprised a mixture of intestine, liver, lung, and heart tissues. Fresh samples were placed in clean sampling bags and packed with ice for transportation to the laboratory. Subsequently, the samples were snap frozen and processed using a meat mincer with a 6 mm mesh. The processed samples were kept at −20 °C until use. Prior to use, the SHW was sterilized at 70 °C in a sealed container in an oven for 1 h. The physiochemical characteristics of the inoculum, CM, PM, and SHW were analyzed before the experiment and are presented in Table 1.

Table 1. Physiochemical characteristics of inoculum and substrates.

Parameter	Inoculum	CM	PM	SHW
pH	8.03	8.21	7.31	7.22
TCOD (g/kg)	12.41	185.73	145.36	1162.64
SCOD (g/kg)	4.30	69.25	35.69	786.74
SCOD/TCOD (%)	34.65	37.29	24.55	67.67
T-N (g/kg)	2.74	4.58	5.23	8.60
NH_4^+-N (g/kg)	1.34	3.21	2.18	1.38
T-P (g/kg)	0.59	5.80	4.22	4.38
TS (g/kg)	17.70	210.02	69.05	305.01
VS (g/kg)	7.10	152.32	54.36	293.14
VS/TS (%)	40.11	72.53	78.73	96.11
C (%)	27.8	33.0	36.7	33.6
H (%)	6.2	5.6	7.1	11.6
O (%)	58.5	51.5	48.5	45.7
N (%)	4.5	2.6	3.8	6.5
S (%)	<0.1	<0.1	1.0	<0.1
C/N ratio	6.2	12.7	9.7	5.2
Chemical formula	$C_{7.26}H_{19.6}O_{11.4}N$	$C_{15.0}H_{30.8}O_{17.6}N$	$C_{11.4}H_{26.5}O_{11.3}N$	$C_{6.0}H_{25.0}O_{6.1}N$
TMY (NmL CH_4/g VS)	-	290.5	358.9	538.8

TCOD: total chemical oxygen demand; SCOD: soluble chemical oxygen demand; T-N: total nitrogen; T-P: total phosphorous; TS: total solids; VS: volatile solids; TMY: theoretical methane yield.

2.2. Experimental Design

The experimental design in this study utilized a mixture design to determine the optimal mixture ratio for CM, PM and SHW. This approach establishes a surface model for continuous variables, allowing the estimation of each component in the mixture and their interactions. The mixtures were combined based on the VS concentration ratios derived from the experimental design (Table 2) and the designed experiments were replicated three times. The response variables, biodegradability (BD), and synergy index (SI) were analyzed using the response surface methodology (RSM), a statistical technique employed for optimization. The RSM examines the relationship between factors (X_1, X_2, ⋯ X_n) and the response (Y) based on their functional relationship. The functional relationship between these factors was analyzed and modeled using analysis of variance (ANOVA) in the Design Expert software. The experimental mixture ratios derived from the mixture design are shown in Table 2.

Table 2. Experimental mixture ratios derived from mixture design.

Standard Order	Mixture Ratio (Based on VS Content)		
	CM (%)	PM (%)	SHW (%)
S1	1	0	0
S2	0	1	0
S3	0	0	1

S4	0.5	0.5	0
S5	0.5	0	0.5
S6	0	0.5	0.5
S7	0.33	0.33	0.33
S8	0.67	0.17	0.17
S9	0.17	0.67	0.17
S10	0.17	0.17	0.67

2.3. Analytical Methods

The pH was measured using a pH meter (Orion star A211, Thermo Scientific, Waltham, MA, USA). TS, VS, TN, and TP were analyzed according to standard methods [18]. TCOD was analyzed using the closed reflux and colorimetric method. SCOD was analyzed by centrifuging the sample at 3000 rpm for 10 min, followed by filtration through a glass microfiber filter (Cytiva Whatman, GF/C, Marlborough, MA, USA), and then applying the same method used for TCOD [19]. The biogas produced was collected in a 100 mL Tedlar bag and quantified in a thermostatic room using a water substitution method. The composition of biogas from the reactors was analyzed using a 6890 N gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with a thermal conductivity detector and coupled to a Shin carbon micro packed column (ResTek, Bellefonte, PA, USA). Helium gas served as the carrier gas for the analysis, with a fixed flow velocity of 6.5 mL/min and inlet and detector temperatures set at 150 °C and 250 °C, respectively. The C, H, O, N, and S contents were determined using an elemental analyzer (TruSpec, Micro CHNS, LECO, Inc., St. Joseph, MI, USA).

2.4. Biochemical Methane Potential Test

The biochemical methane potential (BMP) test was conducted using 250 mL glass bottles with a working volume of 200 mL (Pyrex, Brühl, Germany). Ten substrates were prepared by mixing CM, PM, and SHW based on their VS concentration (see Table 2). The inoculum was preincubated and degassed to deplete any residual biodegradable organic matter before use [20]. Each glass bottle was uniformly set with an inoculum-to-substrate ratio of 3.5 based on the VS content to minimize issues related to acidification or inhibition [21,22]. The culture media was prepared by referring to a recipe from a previous study [23]. Additionally, a 100 mL Tedlar bag (Top Training Eng. Co., Ltd., Incheon, South Korea) was connected to each glass bottle to collect biogas. To create favorable conditions for AD, the pH of all the reactors was initially adjusted to a neutral level (7.5 ± 0.3) by adding 1 N NaOH and 1 N HCl. Furthermore, to prevent a decrease in pH due to acid accumulation, 1.2 g/L of sodium bicarbonate ($NaHCO_3$) was added to each bottle before the test began. Prior to the experiment, all glass bottles were purged with N_2 gas for 3 min to remove oxygen. The glass bottles were then placed in a shaking incubator (Visionbionex, Bucheon-si, South Korea) set at a mesophilic temperature of 35 °C and continuously shaken at 140 rpm throughout the experimental period. Biogas production was measured and analyzed once every two days during the experimental period.

2.5. Calculation

2.5.1. Theoretical Methane Yield

Theoretical methane yield (TMY) refers to the maximum amount of methane that could be produced when all organic matter in the added substrate is completely converted into methane. The calculation of TMY utilizes the results of an elemental analysis of the substrate, following Boyle's reaction equation as indicated in Equation (1).

$$C_aH_bO_cN_dS_e + \left[a - \frac{b}{4} - \frac{c}{2} - \frac{3d}{4} - \frac{e}{2}\right] H_2O$$

$$\left[\frac{a}{2}+\frac{b}{8}-\frac{c}{4}-\frac{3d}{8}-\frac{e}{4}\right]CH_4+\left[\frac{a}{2}-\frac{b}{8}+\frac{c}{4}+\frac{3d}{8}+\frac{e}{4}\right]CO_2+dNH_3+eH_2S \tag{1}$$

2.5.2. Cumulative Methane Yield

The substrate was injected into the glass bottles at a specific concentration, and the amount of biogas collected in the Tedlar bags was quantified every two days during the BMP test. The cumulative methane yield (CMY) was calculated based on the concentration of the injected substrate, taking into account the headspace volume of the glass bottle as described in Equation (2).

$$V_{CH_4}(35℃) = C_1(V_1+V_0) - C_0V_0 \tag{2}$$

where V_{CH4} (35 °C) is the volume of CH_4 under 35 °C (mL); C_1 is methane content at the measurement point; C_0 is the methane content (%) at previous measurement points; V_1 is the biogas volume collected in the Tedlar bags (mL); and V_0 is the biogas volume in the headspace (mL).

The calculated volume of methane was converted to standard conditions using Equation (3).

$$V_{CH_4}(STP) = V_{CH_4}(35℃) \times \frac{273}{273+T} \times \frac{760-P_T}{760} \tag{3}$$

where V_{CH4} (STP) is the volume of CH_4 under standard temperature and pressure (mL); T is the temperature of the glass bottle (35 °C); and P_T is the standard vapor pressure (42 mm H_2O at 35 °C).

2.5.3. Biodegradability

The assessment of biodegradability was conducted by considering the percentage of TMY determined from the elemental composition of the substrate and CMY measured during the BMP test, as indicated in Equation (4).

$$Biodegradability(\%) = \frac{CMY}{TMY} \times 100 \tag{4}$$

where *CMY* is the cumulative methane yield and *TMY* is the theoretical methane yield calculated using the substrate chemical formula's stoichiometric balance.

2.5.4. Synergy Index

The synergy index (*SI*) was evaluated using the *CMY*, and the findings were examined to confirm the synergetic impact of anaerobic co-digestion (AcoD).

$$Synergy\ Index(SI) = \frac{Measured\ CMY}{Calculated\ CMY} \tag{5}$$

The *CMY* values obtained from the AcoD experiments at various ratios were measured, and the calculated *CMY* values were determined by combining the individual *CMY* result based on the mixture ratio. An *SI* value of 1 signifies a straightforward additive effect, while an *SI* less than 1 suggests an antagonistic effect, and an *SI* greater than 1 indicates a synergetic effect.

2.5.5. Kinetic Model

The modified Gompertz model is a type of kinetic model utilized for explaining the total biogas production during AD. In addition to methane yield, the duration of the lag phase (λ) is also a crucial factor for assessing the effectiveness of AD. This can be determined using a modified Gompertz model and Equation (6).

$$M(t) = M_{max} \times exp[-exp\left[\frac{R_{max} \times e}{M_{max}}(\lambda - t) + 1\right]] \quad (6)$$

where *M(t)* is the cumulative methane yield at time t (mL/g VS); M_{max} is the potential maximum methane yield (mL CH_4/g VS); R_{max} is the maximum methane yield rate (mL CH_4/g VS/d); λ is the lag phase (d); t is the duration of the assay (d); and e is 2.7183.

3. Results and Discussion

3.1. Biochemical Methane Potential Test Results

Figure 1 depicts the cumulative methane yield profiles of the BMP runs with varying substrate mixture ratios. The CMY was highest for SHW at 500.2 NmL CH_4/g VS, followed by PM at 264.1 NmL CH_4/g VS and CM at 107.0 NmL CH_4/g VS in anaerobic mono-digestion (AmoD) reactors (S1-S3). Similarly, the BD of the AmoD reactors was 92.8% for SHW, 61.6% for PM, and 30.8% for CM (Table 3), indicating that CM had the lowest CMY and BD compared to PM and SHW. Notably, the CMY of PM closely matched the findings from a previous study, which reported a similar CMY of 238.1 NmL CH_4/g VS. However, the CMY reported for CM in that study was notably higher at 147.4 NmL CH_4/g VS, marking a 38% disparity compared with our results [24]. The difference in CMY for CM is attributed to its characteristics. In South Korea, CM often contains plant-based bedding materials used within livestock facilities, leading to a higher content of non-biodegradable materials such as cellulose, hemicellulose, and lignin. On average, South Korean CM contains 29.2% cellulose, 19.5% hemicellulose, and 13.5% lignin, collectively constituting over 60% of its dry weight [25]. Conversely, CM from the European Union (EU) contains comparatively lower concentrations of cellulose (22.2%), hemicellulose (16.5%), and lignin (15%) [26]. In the case of SHW, the higher CMY and BD can be attributed to its physiochemical characteristics. Notably, its high VS/TS ratio of 96.11% consequently led to a higher methane yield. SHW readily undergoes hydrolysis to produce glycerol and long-chain fatty acids because of its nutrient-rich composition. Furthermore, acetogenic bacteria and methanogenic archaea can efficiently convert these compounds into methane [27]. However, the experimental results from our study (S3) revealed a CMY of 500.2 NmL CH_4/g VS, which appears to be lower than the results from previous studies. This variation in CMY could be attributed to the protein and fat contents of animal tissues. For instance, a previous study reported that the SHW containing a protein of 11.7% and a fat of 38.3% showed a CMY of 702.4 NmL CH_4/g VS [28]. Another study reported a CMY of 639.5 NmL CH_4/g VS using SHW with protein and fat contents of 28.4% and 27.3%, respectively [6]. These findings further affirm the direct correlation between CMY and protein and fat contents within the substrate. Another crucial factor potentially impacting CMY is ammonia inhibition, especially at higher OLRs exceeding 0.8 kg/VS/m^3/d [29]. The lower CMY observed for the SHW in this study may be attributed to two factors. First, the SHW had a lower fat content compared to previous studies. Secondly, the high OLR of 1 kg/VS/m^3/d used in this study may have led to ammonia inhibition, which was not as much of an issue in previous studies that employed lower OLRs to avoid ammonia inhibition.

Table 3. Biodegradability based on the co-digestion mixture ratio.

Standard Order	Mixture Ratio (Based on VS Content)			TMY (NmL CH_4/g VS)	CMY (NmL CH_4/g VS)	BD (%)
	CM	PM	SHW			
S1	1	0	0	347.0	107.0	30.8
S2	0	1	0	428.7	264.1	61.6
S3	0	0	1	538.8	500.2	92.8
S4	0.5	0.5	0	387.9	190.6	49.1
S5	0.5	0	0.5	442.9	356.9	80.6
S6	0	0.5	0.5	483.8	419.9	86.8

S7	0.33	0.33	0.33	433.8	334.8	77.2
S8	0.67	0.17	0.17	397.0	254.7	64.2
S9	0.17	0.67	0.17	437.8	333.9	76.3
S10	0.17	0.17	0.67	492.9	433.1	87.9

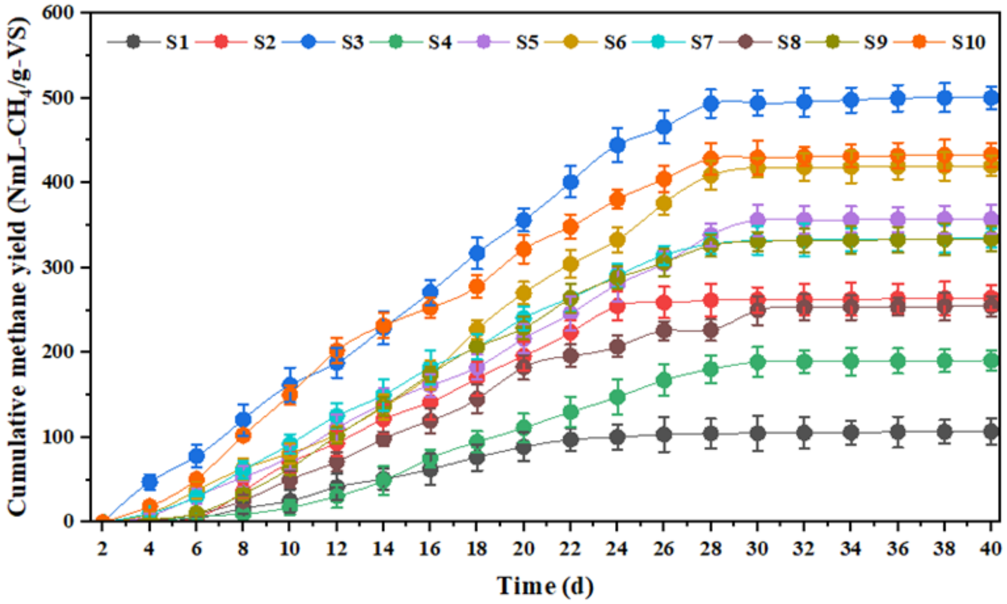

Figure 1. Cumulative methane yield in the biochemical methane potential test.

Likewise, the experimental findings from the AcoD reactors (S4-S10) demonstrated that a higher proportion of SHW in the mixtures led to an increase in both CMY and BD. In particular, reactor S10, which had the highest proportion of SHW, exhibited the highest CMY of 433.1 NmL CH_4/g VS and BD of 87.9% among the AcoD reactors. These findings support a result of previous research that reported a methane yield of 507 mL CH_4/g VS when the CM/SHW mixture ratio was 1:2 based on the VS concentration. The elevated methane yield from the AcoD reactors stems from the synergistic effects of co-digesting SHW with CM and PM. It has been reported that the high protein content in SHW can mitigate pH drops induced by organic acids during the degradation process by generating NH_4^+-N, thereby facilitating stable operation [30]. On the other hand, reactors S8 and S9, which had a lower mixture ratio of SHW, showed lower CMY values of 254.7 NmL CH_4/g VS and 333.9 NmL CH_4/g VS, respectively. A similar result was observed in a previous study, in which a methane yield of 290 NmL CH_4/g VS was achieved from a substrate mixture of SHW and livestock manure at a VS concentration ratio of 1:9 [31]. These results indicate that the beneficial effect of co-digestion primarily relies on the proportion of readily degradable SHW. This promotes microbial growth and enzyme production, aiding in the rapid breakdown of slowly biodegrading organics like CM.

The CMY results obtained from the BMP test were fitted using a modified Gompertz model and the results are presented in Table 4. All R-square values exceeded 0.9, indicating that the modified Gompertz model is suitable for interpreting the CMY data. The R_{max} was highest at 65.3 NmL CH_4/g VS/d in reactor S3 and lowest at 20.2 NmL CH_4/g VS/d in reactor S1. An increase in the mixture ratio of highly biodegradable SHW (0.17, 0.33, 0.5, and 0.67) corresponded to an increase in R_{max} (38.5, 48.2, 58.9, and 61.1 NmL CH_4/g VS/d) in reactors S8, S7, S6, and S10, respectively. This trend is consistent with the findings of a previous study that found a high R_{max} of 42 NmL CH_4/g VS/d for SHW alone, which decreased to 39 NmL CH_4/g VS/d when the SHW was co-digested with livestock manure (LM) at a 0.64:0.36 ratio based on the VS concentration [32]. Another study reported a 30% increase in R_{max} when the SHW mixture ratio was raised to 20% in co-digestion experiments with water hyacinth [33]. The lag phase analysis revealed similar trends to those of

R_{max}. As the proportion of highly biodegradable SHW in the mixture increased, the lag phase decreased. In the AmoD reactors, the shortest lag phase of 3.8 days was observed in reactor S3 with SHW. In contrast, among the AcoD reactors, reactor S10, characterized by a higher proportion of SHW compared to CM and PM, exhibited the shortest lag phase of 3.9 days. However, a previous study reported a shorter lag phase of 2.5 days when the SHW was mixed with LM at a 0.64:0.36 ratio based on the VS concentration [32]. The slight difference in results can be attributed to the varying physiochemical characteristics of CM and PM. Overall, these results suggest that integrating highly biodegradable SHW into co-digestion processes can improve the low R_{max} values and long lag phases associated with CM and PM.

Table 4. Modified Gompertz model fitting results for experimental mixtures.

Standard Order	Mixture Ratio (Based on VS Content)			R_{max} (NmL CH_4/g VS/d)	λ (d)	R^2
	CM	PM	SHW			
S1	1	0	0	20.2	6.7	0.9969
S2	0	1	0	45.2	6.6	0.9931
S3	0	0	1	65.3	3.8	0.9926
S4	0.5	0.5	0	31.4	6.4	0.9817
S5	0.5	0	0.5	45.2	5.9	0.9927
S6	0	0.5	0.5	58.9	5.2	0.9874
S7	0.33	0.33	0.33	48.2	5.2	0.9959
S8	0.67	0.17	0.17	38.5	5.8	0.9949
S9	0.17	0.67	0.17	54.2	5.4	0.9924
S10	0.17	0.17	0.67	61.1	3.9	0.9935

3.2. Results of Response Surface Methodology Model

To evaluate the effects of the substrate mixing ratio more comprehensively, an RSM was generated based on the BMP results. The RSM is particularly effective in situations where the relationship between factors and response values exhibits a curvature, typically represented by a quadratic curve, facilitating the determination of an optimal mixture ratio. However, the results derived from the BMP test, including BD, R_{max}, and the lag phase, displayed a first-order reaction pattern, with all response values increasing as the proportion of SHW as a co-substrate increased. Consequently, the model was developed by calculating BD based on CMY and additionally considering the SI. The calculated SI results for S1–S10 are presented in Figure 2a. The SI for all AmoD conditions was 1, indicating no synergistic effect. On the other hand, the SI for AcoD ranged from 1.03 to 1.26, suggesting that all reactors exhibited a synergistic effect. The highest SI of 1.26 was observed in reactor S8 (CM/PM/SHW = 0.67:0.17:0.17), which had a higher proportion of CM as the co-substrate. In contrast, reactor S4, with an equal mix of CM and PM, showed the lowest SI of 1.03. The SI results for S5 (CM/SHW = 0.5:0.5) and S6 (PM/SHW = 0.5:0.5) indicated that mixing SHW with CM resulted in a higher SI compared to mixing with PM. Previous studies have reported that the primary cause of synergistic effects is attributed to the physicochemical characteristics of the SHW. The AmoD of SHW can act as an inhibitory factor for methanogenic archaea due to the accumulation of VFAs or free ammonia, potentially reducing the methane yield [34]. In contrast, AcoD can enhance synergistic effects through an improved buffer capacity, pH equilibration, nutritional balance, and dilution of inhibitory compounds [35]. According to previous studies, the high nitrogen content in SHW is attributed to its high protein content, which results in a low C/N ratio of approximately 4.09 [36,37]. This characteristic of SHW suggests that an increase in the OLR significantly affects the accumulation of VFAs and the impact of ammonia [36]. Other research has also reported that even at a low OLR of 0.8 kg/VS/m^3/d, the methane yield is reduced due to ammonia inhibition [29]. The low C/N ratio of SHW can be improved by mixing with substrates high in lignocellulosic content [38]. In particular, livestock manure predominantly composed of lignocellulose, polysaccharides, proteins, and other organic materials are ideal as co-substrates [39]. The study results indicated that the C/N ratios for CM, PM, and SHW were 12.7, 9.7, and 5.2, respectively, with SHW having the lowest value. This suggests that the relatively higher C/N ratio of CM could contribute to an increased synergistic effect as its content increases. The results indicated that the model's contour and 3D surface plots showed similar trends. BD was observed to increase with the rising content of SHW. The SI was found to increase with higher amounts of livestock manure, with a particularly noticeable rise as the content of CM increased. Consequently, this information was utilized to derive the optimal mixture ratio for each substrate component.

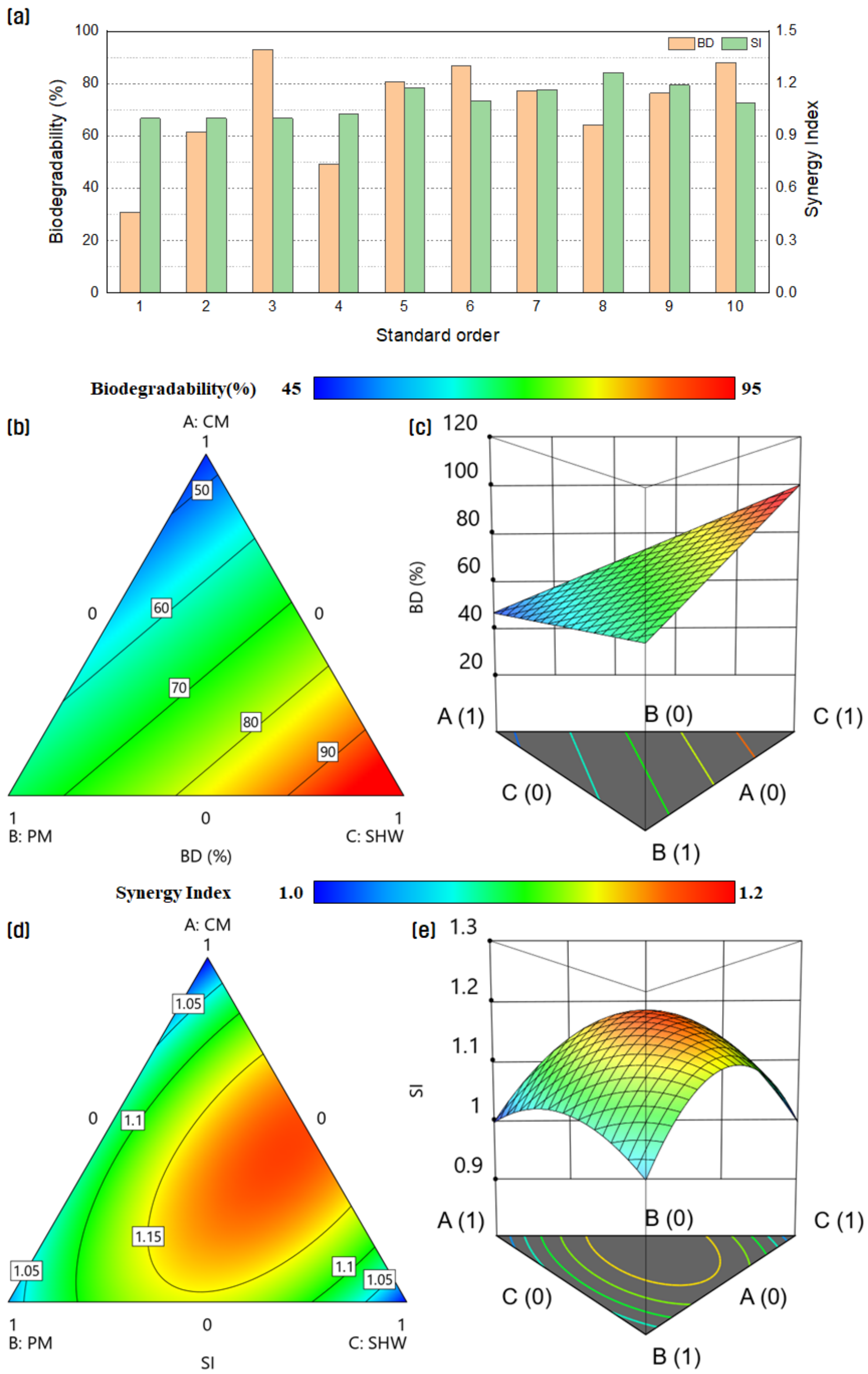

Figure 2. Biodegradability and synergy index (**a**), contour plots (**b**,**c**), 3D surface plots (**d**,**e**).

3.3. Optimal Substrate Mixture Ratio

The optimal mixture ratio of CM, PM, and SHW was derived using the BD and SI data. A Piepel trace plot (Figure 3a,b) allowed us to examine the changes in the dependent variables (BD, SI) with the variation in the mixture ratios. Similar to a main effect plot, the Piepel trace plot showed how changes in mixture components affects the responses. However, in mixture design, adjusting the proportion of one ingredient necessitates corresponding changes in others to maintain a total sum of one—a unique constraint of mixture designs. Steeper slopes on the Piepel trace plot indicate a stronger impact of a component's proportion change on the response. Figure 3a reveals that as the proportion of SHW increased, BD also increased, and the steepness of the slope suggests that SHW had the most significant effect on BD. Conversely, as the proportions of CM and PM increased, BD decreased, with CM having a more pronounced effect on BD than PM. Figure 3b illustrates the influence of the mixture ratio on SI, indicating that both SHW and CM had a significant impact, like their effects on BD. Changes in the SHW proportion resulted in the most pronounced SI shifts, while alterations in the CM proportion had a greater impact compared to PM.

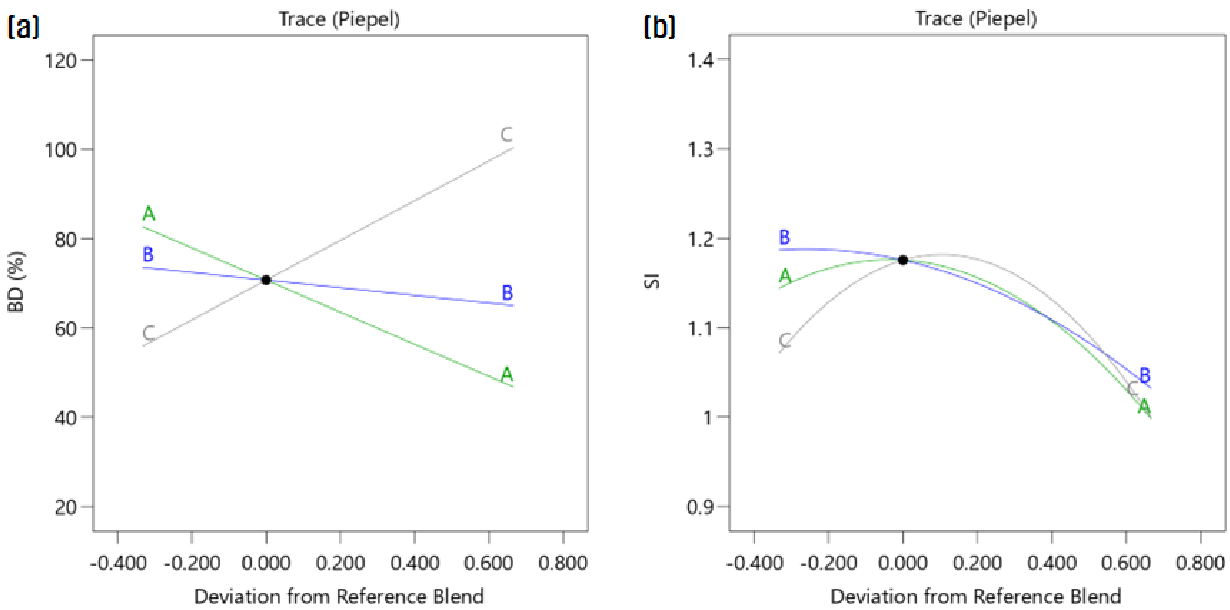

Figure 3. Piepel trace plot of biodegradability (**a**) and synergy index (**b**) (A: CM; B: PM; C: SHW).

The derivation of the optimal mixture ratio using a model can vary depending on the desired response value. Figure 3b suggests that the optimal mixture ratio corresponds to S3, where BD increased with higher proportions of SHW. Conversely, using the SI results from Figure 2c, the optimal mixture ratio aligned with S8. However, relying solely on one response value to determine the optimal mixing ratio may introduce bias towards specific factors. Therefore, by using both the BD and SI response values, an overlay plot was utilized to derive the optimal mixture ratio, as shown in Figure 4a. At the same mixture ratio, the BD and SI values were 70.7 and 1.17, respectively. Taking these as a baseline, the optimal range up to a BD of 75% and an SI of 1.18 is indicated by the yellow area in the overlay plot. Thus, depending on the objective, the optimal mixture ratio that maximizes both response values was 0.27:0.31:0.42 (CM/PM/SHW), while the optimal mixture ratio that maximizes the content of CM was 0.38:0.27:0.36 (CM/PM/SHW). To verify the accuracy of the constructed model, the BMP test (Figure 4b) and the BD and SI (Figure 4c) were compared and analyzed. The analysis showed that the percentage error for all response values was within 5%, confirming that the constructed model is suitable for predicting the response values.

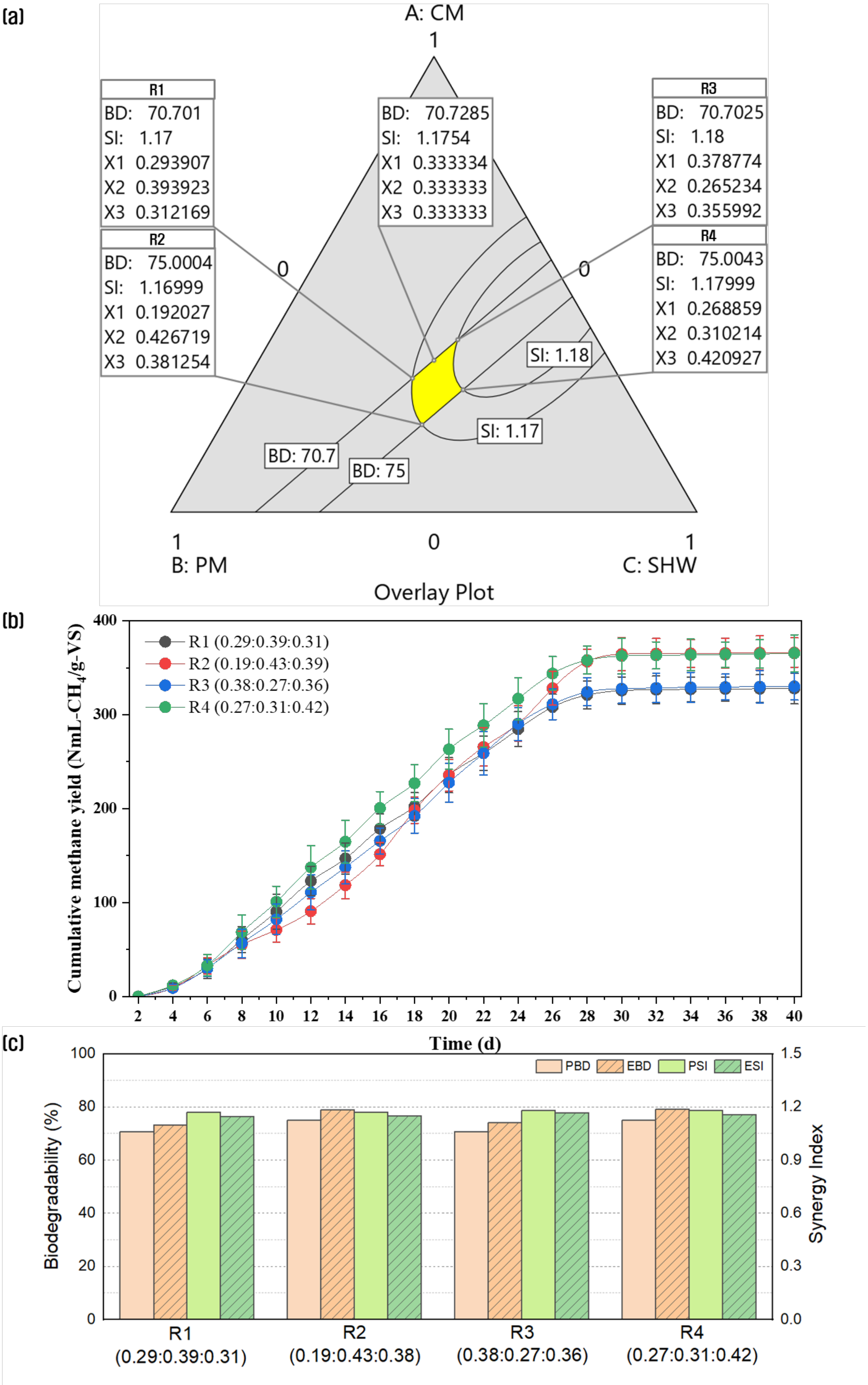

Figure 4. Overlay plot of co-digestion (**a**), biochemical methane potential test (**b**), and predicted and experimental biodegradability and synergy index (**c**).

4. Conclusions

This study demonstrated that the AcoD of SHW can significantly enhance the efficiency of anaerobic digestion of livestock manure. Utilizing Design Expert for the quantitative analysis, we ascertained the synergistic effects based on varying mixture ratios of livestock manure. The synergy was most pronounced when SHW was mixed with CM, which has a higher C/N ratio compared to PM, thereby maximizing the AcoD efficiency of SHW. The results indicated that when CM, PM, and SHW were optimally mixed, the BD and SI reached 70.7% and 1.17, respectively. This proves that alterations in the mixture ratio alone can enhance BD and SI. The model constructed from these results suggested that a mixture ratio of CM/PM/SHW = 0.29:0.39:0.31 achieved a comparable AcoD efficiency, BD, and SI. Furthermore, a mixture ratio of 26.9% CM, 31.0% PM, and 42.1% SHW optimized the biodegradability and synergistic effects. In conclusion, co-digestion with SHW can significantly enhance the BD of livestock manure in AD systems. This improvement depends on identifying the optimal mixture ratio, which can be effectively determined using mixture design techniques in design of experiment software.

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