Optimization Of Amylase-Assisted Anaerobic Co-Digestion of Poultry Droppings, Pig Manure, And Brewery Spent Grain for Enhanced Biogas Production: A Comparative Study of Response Surface Methodology and Adaptive Neuro-Fuzzy Inference System.

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***Abstract*—Biogas was generated via oxygen-independent fermentation of poultry droppings, pig manure, brewery spent grain, and their co-fermentation, with amylase as a promotusing an integrated Response Surface Methodology (RSM) and Adaptive Neuro-Fuzzy Inference System (ANFIS) approach. Physicochemical characterization of the was waste carried out to determine the range of anaerobic digestion (AD). One-Factor-at-a-Time (OFAT) preliminary experiments established feasible ranges for four independent variables: temperature (30–60°C), residence time (10–40 days), enzyme concentration (0–10 µmol/L), and pH (5–9). A Central Composite Design (CCD) with 30 experimental runs was employed for RSM optimization, yielding a highly significant second-order polynomial model ($R^2 = 0.965$, $p < 0.0001$). Enzyme concentration emerged as the most influential factor ($F = 174.2$), followed by residence time, temperature, and pH. Significant interaction effects were observed between temperature and enzyme concentration ($p = 0.006$), residence time and enzyme concentration ($p = 0.019$), and pH and enzyme concentration ($p = 0.034$), demonstrating the synergistic benefits of co-digestion. The ANFIS model, utilizing a hybrid learning algorithm with Gaussian membership functions and 81 fuzzy rules, demonstrated superior predictive accuracy compared to RSM, with R^2 values of 0.983 (training), 0.971 (validation), and 0.967 (testing), alongside lower RMSE (14.2 mL/g VS) and MAPE (3.8%). Under optimized conditions, individual feedstocks yielded 412, 385, and 298 mL/g VS for PD, PM, and BSG, respectively, while co-fermentation in a 1:1:1 ratio achieved a maximum yield of 568 mL/g VS at 50°C, 20 days residence time, 6 µmol/L enzyme concentration, and pH 8 a 42.7% increase over mono-digestion. Validation experiments confirmed model predictions with high accuracy ($p > 0.05$ for paired t-tests). The findings contribute to addressing organic waste management challenges in developing regions while advancing decentralized biogas production technology.**

***Index Terms*—Anaerobic digestion, biogas optimization, co-fermentation, amylase enzyme, One-Factor-at-a-Time, response surface methodology, adaptive neuro-fuzzy inference system, poultry droppings, pig manure, brewery spent grain.**

I. INTRODUCTION

The escalating global energy demand, projected to increase by nearly 50% by 2050, coupled with the intensifying environmental consequences of fossil fuel combustion including greenhouse gas emissions, air pollution, and climate change has compelled an urgent transition toward sustainable and renewable energy sources [11]; [31]. Among the array of renewable energy technologies, anaerobic digestion (AD) stands out as a dual-purpose solution that simultaneously addresses organic waste management challenges and decentralized energy generation [2]; [29]. Biogas, the primary product of AD, is a methane-rich gas (typically 50–70% CH_4) that can be utilized for heating, electricity production, and, after upgrading, as a vehicle fuel or grid-injected biomethane [34].

Developing countries, particularly in sub-Saharan Africa and Southeast Asia, generate vast quantities of organic wastes from agricultural, livestock, and industrial activities. Poultry droppings, pig manure, and brewery spent grain (BSG) are among the most abundant yet underutilized waste streams in these regions [17]; [21]. Indiscriminate disposal of these wastes leads to severe environmental pollution, including eutrophication of water bodies, emission of greenhouse gases (methane and nitrous oxide) from open decomposition, and proliferation of disease vectors [14]. Paradoxically, these wastes are rich in biodegradable organic matter, making them ideal substrates for AD. Poultry droppings contain high nitrogen content, pig manure offers a balanced nutrient profile, and brewery spent grain is lignocellulosic but rich in carbohydrates [20]; [36].

However, mono-digestion of these individual substrates often suffers from suboptimal biogas yields due to inherent limitations.

Rajagopal [27] in their research study on poultry droppings, indicates that poultry droppings exhibit high total ammonia nitrogen (TAN) concentrations that can inhibit methanogenic archaea when accumulated.

Molinuevo-Salces [18] addressed that pig manure, while widely used, has relatively low carbon-to-nitrogen (C/N) ratios, leading to ammonia inhibition and poor process stability).

Panjičko [23] specifically stated in their research study that brewery spent grain possesses a high C/N ratio but is recalcitrant to hydrolysis due to its lignocellulosic structure, making the initial breakdown step rate-limiting.

Mata-Alvarez [16] and Hagos [10] on simultaneous co-digestion, reportedly explored the AD of two or more substrates.it was discovered that the simultaneous co-digestion ,demonstrated to overcome these limitations by balancing nutrients, diluting inhibitory compounds, and improving synergistic microbial activity ,co-fermentation of poultry droppings, pig manure, and BSG can potentially optimize the C/N ratio, enhance buffer capacity, and increase overall biogas yield.

Vavilin [32] on AD of complex organic substrates, stated that the rate-limiting step in AD of complex organic substrates, particularly those rich in carbohydrates and lignocellulose, is hydrolysis, the enzymatic breakdown of polymers into soluble monomers. Exogenous enzyme supplementation has emerged as a promising strategy to accelerate hydrolysis and improve biogas production [24]. Among hydrolytic enzymes, α-amylase catalyzes the cleavage of internal α-1,4-glycosidic bonds in starch, generating fermentable sugars that are readily utilized by acidogenic bacteria [9]. Several studies have reported enhanced biogas yields following amylase addition to starch-rich feedstocks [13]; [8]. Nevertheless, the optimal concentration of amylase, as well as its interactive effects with critical process parameters such as temperature, pH, and residence time, remains insufficiently characterized for mixed agricultural and industrial wastes.

Mao [15] conducted a comprehensive study on Anaerobic digestion. In their study, it was discovered that Anaerobic digestion is a complex, non-linear, and multi-parameter process. The performance of AD systems is influenced by temperature, pH, organic loading rate (OLR), hydraulic retention time (HRT), substrate composition, and enzyme concentration, among others.

Bezerra [5] on their research study on optimization approaches specifically stated that the traditional one-factor-at-a-time (OFAT) optimization approaches are not only time-consuming and resource-intensive but also incapable of detecting interaction effects between variables. Response Surface Methodology (RSM) has been widely adopted as a statistical optimization tool that enables the evaluation of main effects, interaction effects, and quadratic effects using a minimal number of experimental runs [19]. RSM has been successfully applied to optimize biogas production from various organic wastes [6]; [33]. However, RSM is inherently a polynomial regression technique that may not fully capture highly non-linear dynamics inherent in biological systems [35].

Ghatak [7] In their profound studies on artificial intelligence (AI)-based models, affirms that artificial intelligence (AI)-based models have gained traction in bioprocess engineering due to their ability to learn complex, non-linear relationships from experimental data without requiring prior knowledge of underlying mechanisms. The Adaptive Neuro-Fuzzy Inference System (ANFIS), a hybrid intelligent system that integrates the learning capability of neural networks with the reasoning framework of fuzzy logic, has demonstrated superior predictive accuracy compared to RSM in several bioprocess optimization studies [22]; [4]. ANFIS can approximate any continuous function and is particularly suited for processes with uncertainty, vagueness, or incomplete mechanistic understanding [12]. Comparative studies between RSM and ANFIS for biogas production are, however, scarce, especially for enzyme-assisted co-digestion of multiple feedstocks.

Despite the individual merits of RSM and ANFIS, no previous study has systematically integrated both methodologies to optimize amylase-assisted biogas generation from the co-fermentation of poultry droppings, pig manure, and brewery spent grain. Furthermore, comprehensive physicochemical characterization and crystallographic phase analysis of these substrates essential for understanding substrate degradability and model development are often neglected in existing literature [28]. X-ray diffraction (XRD) analysis, for instance, can reveal the crystalline structure of cellulose and other polymers, which directly correlates with hydrolytic susceptibility [25].

Therefore, the present study aims to address these research gaps by: (i) characterizing the physicochemical properties and crystalline phases of poultry droppings, pig manure, brewery spent grain, and their mixtures; (ii) optimizing process parameters (temperature, residence time, enzyme concentration, and pH) for amylase-assisted biogas production from both individual and combined feedstocks using batch experiments; (iii) developing predictive models using RSM and ANFIS. By integrating classical statistical optimization with an intelligent neuro-fuzzy approach, this study provides a robust framework for enhancing biogas productivity from underutilized organic wastes, thereby contributing to sustainable waste management and renewable energy generation in alignment with the circular economy paradigm.

II. MATERIALS AND METHODS

Collection of Samples

Fresh Poultry droppings and pig manure were sourced from Phinomar Farms in Ngwo-Asaa in Enugu State. The brewery's spent grains were sourced from Ibukris Farms Ltd in Obeagu Amaechi in Enugu State. Anaerobic inoculum was sourced from a mesophilic biogas digester operating at MJ BIO Digester Service, Enugu state Nigeria, which had been fed with a mixture of cow dung and food waste for over six months. The enzyme used in this study was commercial α-amylase (EC 3.2.1.1) derived from "Bacillus licheniformis", purchased from A-Matrix Nigerian limited, Yaba Lagos state Nigeria. Other materials and reagents used were sourced from MET department, PRODA Enugu State, Nigeria.

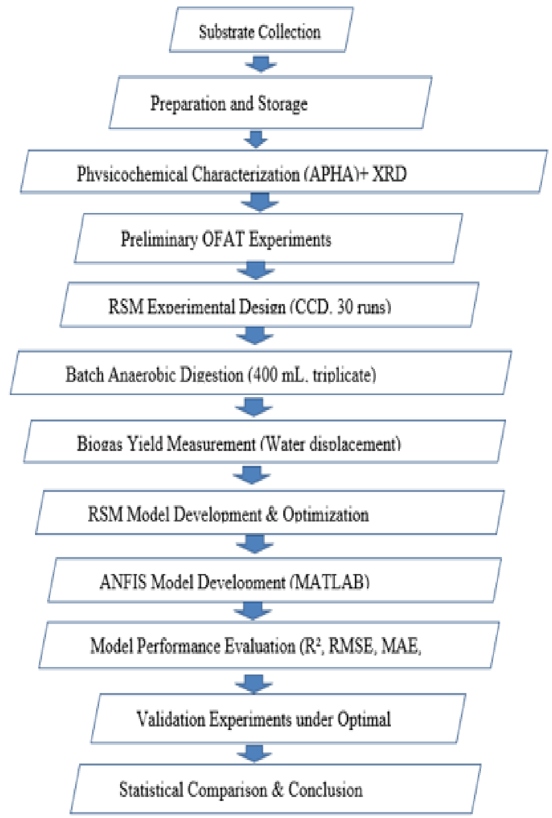

Figure 1: Overall Experimental Flowchart

Characterization of Inoculum

The inoculum was screened through a 1 mm sieve to remove large particles and degassed at 37°C for 5 days to reduce background biogas production. The characteristics of the inoculum are presented below;

Table 1: Characteristics of Anaerobic Inoculum

Parameter	Value ± SD
pH	7.2 ± 0.2
Total Solids (TS, %)	6.8 ± 0.3
Volatile Solids (VS, % of TS)	68.5 ± 2.1
Alkalinity (mg $CaCO_3$/L)	2850 ± 150
Total Ammonia Nitrogen (mg/L)	350 ± 45

The α-amylase enzyme which had a declared activity of 1200 U/mL (one unit defined as the amount of enzyme liberating 1 µmol of reducing sugar per minute at 60°C and pH 6.0). Stock solutions of varying concentrations (0, 2, 4, 6, 8, 10 µmol/L) were prepared fresh daily by diluting the enzyme concentrate with phosphate buffer (0.1 M, pH 7.0). Enzyme solutions were stored at 4°C and used within 24 hours to prevent activity loss.

Physicochemical Analysis (APHA Methods)

All physicochemical analyses were performed in triplicate following standard methods outlined in the American Public Health Association (APHA) Standard Methods for the Examination of Water and Wastewater, 23rd Edition [3]. The following parameters were determined.

Table 2: X-ray Diffraction (XRD) Analysis

Parameter	Method	APHA Reference
pH	Electrometric (pH meter)	4500-H^+ B
Total Solids (TS)	Gravimetric (103–105°C)	2540 B
Volatile Solids (VS)	Gravimetric (550°C)	2540 E
Moisture Content	Gravimetric difference	2540 B
Ash Content	Gravimetric (550°C)	2540 E
Chemical Oxygen Demand (COD)	Closed reflux colorimetric	5220 D
Biochemical Oxygen Demand (BOD_5)	5-day incubation (20°C)	5210 B
Total Kjeldahl Nitrogen (TKN)	Kjeldahl digestion/titration	4500-N org B
Ammonia Nitrogen (NH_3-N)	Phenate method	4500-NH_3 F
Total Phosphorus (TP)	Ascorbic acid method	4500-P E
Total Potassium (K)	Flame photometry	3500-K B
Total Organic Carbon (TOC)	Walkley-Black method	5310 B
Carbon-to-Nitrogen (C/N) Ratio	Calculated (TOC/TKN)	—
Lignin, Cellulose, Hemicellulose	Van Soest detergent fiber method	—
Total Reducing Sugars	DNS (3,5-dinitrosalicylic acid) method	—
Alkalinity	Titration (pH 4.5 endpoint)	2320 B
Volatile Fatty Acids (VFAs)	Distillation/titration	5560 D

The obtained diffractograms were processed using OriginPro. The crystallinity index (CrI) of lignocellulosic components was calculated using the Segal method [30]:

$$CrI\ (\%) = [(I_{002} - I_{am}) / I_{002}] \times 100$$

Where: I_{002} = Maximum intensity of the crystalline peak (2θ ≈ 22°–23°) and

I_{am} = Intensity of the amorphous peak (2θ ≈ 18°–19°).

Experimental Design for Batch Anaerobic Digestion

Batch anaerobic digestion experiments were conducted using 500 mL glass serum bottles (working volume: 400 mL) as digesters. Each bottle was equipped with a rubber septum and screw cap to maintain anaerobic conditions. Biogas collection was achieved using the water displacement method, with graduated glass cylinders inverted in a water bath containing acidified brine (pH < 3 with H_2SO_4) to prevent CO_2 dissolution [1].

Figure 2: Illustration of experimental Set-up.

Each digester was loaded with: Substrate (individual or mixed) to achieve a VS concentration of 10 g/L, Inoculum (10% v/v of working volume, approximately 40 mL), Enzyme solution (according to experimental design) and Distilled water to make up the remaining volume.

The initial pH of each digester was adjusted using 1 M NaOH or 1 M HCl as required by the experimental design.

Two categories of substrate feeding were investigated Category A - Poultry droppings (PD) only, Pig manure (PM) only and Brewery spent grain (BSG) only (Individual Feedstocks) and Category B - Combined Feedstock (Co-fermentation) PD : PM : BSG in a 1:1:1 ratio (by VS basis). The 1:1:1 ratio was selected based on preliminary trials and literature recommendations for co-digestion of agricultural and industrial wastes [10].

Experiments were conducted under mesophilic conditions (temperature range: 30–60°C as per experimental design). Each digester was manually shaken once daily for 1 minute to ensure homogeneity and prevent scum formation. Biogas production was measured daily for the designated residence time (10–40 days). All experiments were performed in triplicate, and results are reported as mean ± standard deviation as seen in Table 3.

Table 3: Proportion of Biomass Waste and Water Used.

Biomas Waste	Weight of Waste (g)	Vol. of Water used (L)
Poultry Droppings	100	1.0
Pig Manure	100	1.0
Brewery Spent Grains	100	1.0
Co-Digestion		
PD: PG: BSG	33.3 g each	1.0

Table 4: Iindependent variables

Variable	Symbol	Unit	Low Level (-1)	Center Point (0)	High Level (+1)
Temperature	X_1	°C	30	45	60
Residence Time	X_2	Days	10	25	40
Enzyme Concentration	X_3	µmol/L	0	5	10
pH	X_4	—	5	7	9

Four independent variables were investigated;

The response variable (Y) was cumulative biogas yield expressed in mL/g VS added.

One-Factor-at-a-Time (OFAT) Preliminary Experiments

Prior to RSM optimization, preliminary OFAT experiments were conducted to identify feasible ranges for each variable. In the OFAT experiments, one variable was varied while holding others at baseline (temperature 45°C, residence time 25 days, enzyme concentration 5 µmol/L, pH 7).The range for each variable was refined based on biogas yield response and Results informed the selection of low, center, and high levels for RSM design.

Response Surface Methodology (RSM) Design for Biogas production

The generalized second-order polynomial model used for RSM is:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j + \varepsilon$$

Where:Y = Predicted biogas yield, β_0 = Intercept coefficient, β_i = Linear coefficients, β_{ii} = Quadratic coefficients, β_{ij} = Interaction coefficients, X_i, X_j = Independent variables and ε = Random error.

For each of the 30 RSM runs (per feedstock category), digesters were prepared according to the specific combination of temperature, residence time, enzyme concentration, and pH prescribed by the CCD. Biogas yield was recorded at the end of the designated residence period.

Adaptive Neuro-Fuzzy Inference System (ANFIS) Modeling of Biogas

ANFIS is a hybrid intelligent system that integrates a Takagi-Sugeno-type fuzzy inference system with a five-layer neural network architecture [12].

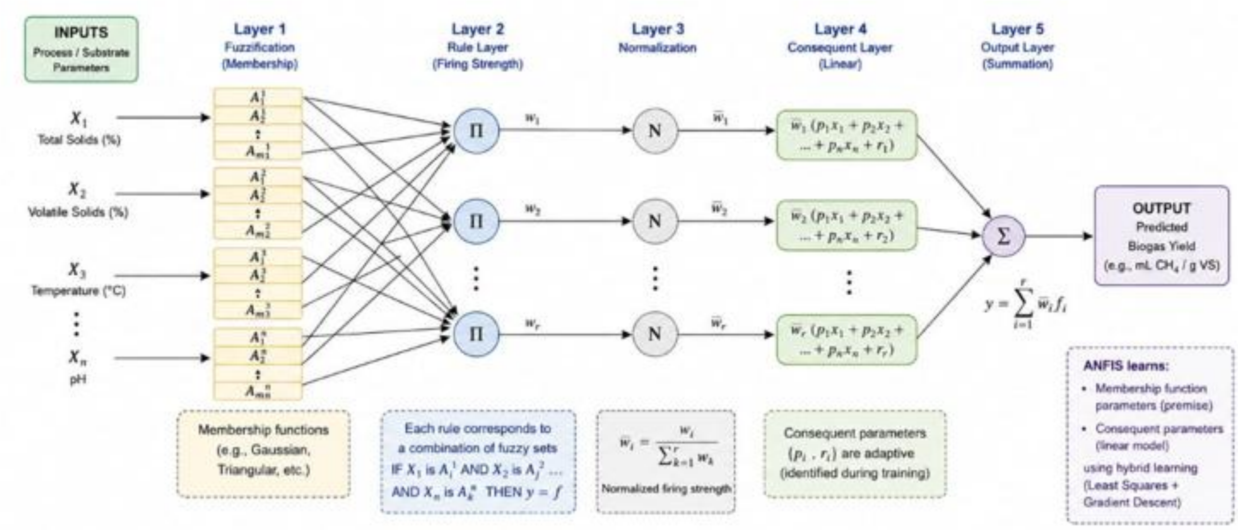

Figure 3: ANFIS Architecture for Biogas Yield Prediction

Layer 1 (Input Fuzzification): Each input variable (temperature, residence time, enzyme concentration, pH) was assigned membership functions (MFs). Gaussian membership functions were used:

$\mu(x) = \exp[-(x - c)^2 / 2\sigma^2]$

Layer 2 (Rule Firing): Calculates the firing strength (w_i) of each rule as the product of input membership degrees.

Layer 3 (Normalization): Normalizes firing strengths: $\bar{w}_i = w_i / \Sigma w_j$

Layer 4 (Defuzzification): Computes rule outputs using first-order Sugeno model: $\bar{w}_i(p_i x + q_i y + r_i)$

Layer 5 (Output Summation): Sums all rule outputs: $Y = \Sigma \bar{w}_i f_i$

ANFIS Data Preparation, Partitioning and Model Development for biogas.

The experimental data (30 runs per feedstock category) were divided into Training Validation and Testing as seen in Table 5.

Table 5: Experimental Data

Dataset	Percentage	Number of Samples	Purpose
Training	70%	21	Model parameter estimation
Validation	15%	5-Apr	Model selection and tuning
Testing	15%	5-Apr	Independent performance

Data were randomized prior to partitioning to avoid ordering bias. Inputs and output were normalized to the range [0, 1] using min-max normalization:

X_norm = (X - X_min) / (X_max - X_min)

ANFIS modeling was performed using the ANFIS Editor in MATLAB R2023a (The MathWorks, Inc., Natick, MA, USA).

Table 6: ANFIS Parameter Settings

Parameter	Setting
Number of inputs MFs	3 (low, medium, high) per input
MF type	Gaussian (gaussmf)
Output MF type	Linear (Sugeno type)
Optimization method	Hybrid (backpropagation + least squares)
Number of epochs	100
Error tolerance	0.00001
Initial step size	0.01
Step size decrease rate	0.9
Step size increase rate	1.1

The fuzzy inference system (FIS) was generated using grid partitioning due to the moderate number of inputs (4). The totalnumber of fuzzy rules generated was 3^4 = 81 rules.

Model Training and Validation for biogas

During training, the hybrid learning algorithm updated:

Premise parameters (MF centers and widths) via backpropagation and Consequent parameters (polynomial coefficients) via least squares estimation.Training continued for 100 epochs or until the validation error ceased to decrease (early stopping). Model performance during training was monitored using root mean square error (RMS

The predictive accuracy of both RSM and ANFIS models was evaluated using the following statistical metrics:

Coefficient of Determination (R^2)

$R^2 = 1 - [\Sigma(Y_{exp} - Y_{pred})^2 / \Sigma(Y_{exp} - \bar{Y}_{exp})^2]$

R^2 indicates the proportion of variance in the experimental data explained by the model (range: 0 to 1; higher is better).

Adjusted R^2 (R^2_{adj})

$R^2_{adj} = 1 - [(1 - R^2)(n - 1) / (n - p - 1)]$

Where n = number of observations, p = number of predictors. Penalizes model complexity.

Root Mean Square Error (RMSE)

$RMSE = \sqrt{[\Sigma(Y_{exp} - Y_{pred})^2 / n]}$

Measures average prediction error in the same units as biogas yield (mL/g VS).

Mean Absolute Error (MAE)

$MAE = \Sigma|Y_{exp} - Y_{pred}| / n$

Less sensitive to outliers than RMSE.

Mean Absolute Percentage Error (MAPE)

$MAPE = (100/n)\ \Sigma|(Y_{exp} - Y_{pred}) / Y_{exp}|$

Expresses prediction error as a percentage.

Nash-Sutcliffe Efficiency (NSE)

$NSE = 1 - [\Sigma(Y_{exp} - Y_{pred})^2 / \Sigma(Y_{exp} - \bar{Y}_{exp})^2]$

Range: $-\infty$ to 1; NSE = 1 indicates perfect prediction.

Model Validation Experiments

To validate the optimal conditions predicted by RSM and ANFIS, additional batch experiments were conducted in triplicate under the following conditions:

For Individual Feedstocks; Temperature: 42.5°C, Residence time: 30 days, Enzyme concentration: 4 μmol/L and pH: 7.

For Combined Feedstock (1:1:1 PD:PM:BSG); Temperature: 50°C, Residence time: 20 days, Enzyme concentration: 6 μmol/L and pH: 8.

The experimentally obtained biogas yields were compared with model predictions using paired t-tests ($\alpha = 0.05$) to determine if significant differences existed.

Statistical Analysis

All statistical analyses were performed using R version 4.2.2 [26] and Minitab 19. The following statistical procedures were applied:

Table 7: Statistical Analysis

Analysis	Method	Software
Descriptive statistics	Mean, SD, SEM	R, Excel
Comparison of means	One-way ANOVA + Tukey HSD	R
RSM regression	Multiple linear regression (CCD)	Design-Expert
RSM significance testing	ANOVA with F-test	Design-Expert
Model comparison	Paired t-test, RMSE, R^2	R
Normality of residuals	Shapiro-Wilk test	R
Homogeneity of variance	Levene's test	R
Outlier detection	Cook's distance	R

Significance level: $\alpha = 0.05$ ($p < 0.05$ considered statistically significant).

III. RESULTS AND DISCUSSION

The physicochemical properties of poultry droppings (PD), pig manure (PM), brewery spent grain (BSG), and their 1:1:1 mixture (on VS basis) are presented in Table 8. All analyses were performed in triplicate, and results are expressed as mean ± standard deviation.

Table 8: Physicochemical Characteristics of Substrates (Mean ± SD, n = 3)

Parameter	Poultry Droppings	Pig Manure	Pig Manure	Brewery Spent Grains	Mixed (1:1:1)
Ph	7.8 ± 0.3	7.2 ± 0.2	7.2 ± 0.2	5.4 ± 0.2	6.8 ± 0.2
Moisture Content (%)	74.6 ± 1.5	81.4 ± 1.2	81.4 ± 1.2	77.7 ± 1.4	78.8 ± 1.1
Total Solids (TS, %)	25.4 ± 1.2	18.6 ± 0.9	18.6 ± 0.9	22.3 ± 1.1	21.2 ± 0.8
Volatile Solids (VS, % TS)	78.5 ± 2.1	82.3 ± 1.8	82.3 ± 1.8	96.2 ± 1.5	85.7 ± 1.6

Ash Content (% TS)	21.5 ± 1.8	17.7 ± 1.5	17.7 ± 1.5	3.8 ± 0.4	14.3 ± 1.2
COD (g/L)	85.4 ± 3.2	72.6 ± 2.8	72.6 ± 2.8	112.4 ± 4.1	90.1 ± 3.5
BODS (g/L)	42.3 ± 2.1	38.7 ± 1.9	38.7 ± 1.9	58.2 ± 2.5	46.4 ± 2.2
TKN (g/kg TS)	42.5 ± 1.8	35.2 ± 1.5	35.2 ± 1.5	18.6 ± 0.9	32.1 ± 1.4
NH_3-N (g/kg TS)	8.6 ± 0.5	6.4 ± 0.4	6.4 ± 0.4	1.2 ± 0.1	5.4 ± 0.3
Total Phosphorus (g/kg TS)	18.4 ± 0.9	14.2 ± 0.7	14.2 ± 0.7	3.8 ± 0.2	12.1 ± 0.6
Total Potassium (g/kg TS)	22.6 ± 1.1	16.8 ± 0.8	16.8 ± 0.8	1.5 ± 0.1	13.6 ± 0.7
Total Organic Carbon (g/kg TS)	348 ± 12	372 ± 14	372 ± 14	538 ± 18	419 ± 15
C/N Ratio	8.2 ± 0.4	10.5 ± 0.5	10.5 ± 0.5	28.6 ± 1.2	15.8 ± 0.7
Alkalinity (mg $CaCO_3$/L)	3250 ± 180	2850 ± 150	2850 ± 150	1250 ± 100	2450 ± 140
Total VFAs (mg/L as acetic)	1850 ± 120	1620 ± 110	1620 ± 110	420 ± 35	1297 ± 88

pH Values: The pH of PD (7.8) and PM (7.2) were within the neutral to slightly alkaline range, which is favorable for methanogenic activity [15]. In contrast, BSG exhibited an acidic pH (5.4), consistent with residual organic acids from the brewing process and partial lactic acid fermentation during storage [23]. The mixed feedstock achieved a near-neutral pH of 6.8, demonstrating the buffering benefit of co-digestion. This observation aligns with [10], who reported that co-digestion of acidic industrial wastes with alkaline livestock manures stabilizes pH without external chemical addition.

Volatile Solids: BSG showed the highest VS content (96.2% TS), indicating its high organic matter content and therefore high biogas potential. However, as discussed later, the high lignocellulose content limits its biodegradability despite the high VS. PD (78.5% TS) and PM (82.3% TS) had moderate VS contents, comparable to values reported by [17] and[18].

C/N Ratio: The C/N ratios of PD (8.2) and PM (10.5) were below the optimal range for anaerobic digestion (15–30), indicating nitrogen excess that could lead to ammonia inhibition [27]. BSG exhibited a high C/N ratio (28.6), reflecting carbon excess due to its carbohydrate-rich composition. The mixed feedstock achieved a C/N ratio of 15.8, which falls within the optimal range. This balance is a key advantage of co-digestion, as it reduces the risk of ammonia toxicity while providing sufficient carbon for methanogenesis. Wang [33] similarly reported that co-digestion of nitrogen-rich manures with carbon-rich agricultural residues optimizes the C/N ratio and enhances biogas yield.

COD and BOD_5: BSG exhibited the highest COD (112.4 g/L) and BOD_5 (58.2 g/L), reflecting its high organic strength. However, the BOD_5/COD ratio of BSG (0.52) was lower than that of PD (0.50) and PM (0.53), suggesting a higher proportion of recalcitrant organic matter. The mixed feedstock showed intermediate values with a BOD_5/COD ratio of 0.51, indicating good biodegradability.

Nutrient Content: PD contained the highest concentrations of nitrogen (TKN: 42.5 g/kg TS), phosphorus (18.4 g/kg TS), and potassium (22.6 g/kg TS), reflecting the concentrated nature of poultry excreta. While beneficial as a fertilizer, high nitrogen levels can be inhibitory in AD. BSG had the lowest nutrient levels, consistent with its plant-based origin. The mixed feedstock provided a more balanced nutrient profile for microbial growth.

Alkalinity and VFAs: PD showed the highest alkalinity (3250 mg $CaCO_3$/L), which provides buffering capacity against pH drops. BSG had low alkalinity (1250 mg $CaCO_3$/L) but also low VFAs (420 mg/L), indicating limited acidogenic activity. The mixed feedstock achieved sufficient alkalinity (2450 mg $CaCO_3$/L) to resist pH fluctuations during digestion.

X-ray Diffraction (XRD) Analysis

The X-ray diffractograms of PD, PM, BSG, and the mixed feedstock are presented in Figures 4. Table 4.2 summarizes the characteristic peak positions (2θ) and corresponding crystalline phases.

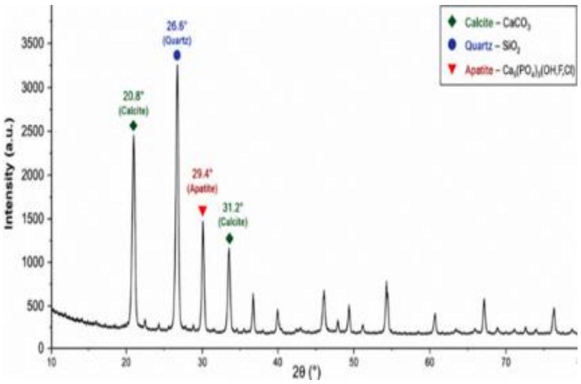

Figure 4.1: XRD of Substrate Poultry Droppings.

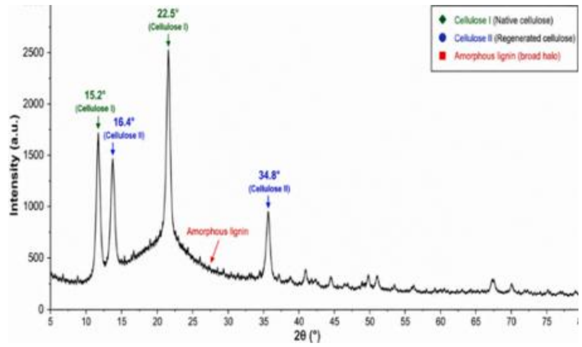

Figure 4.3: XRD of Brewery Spent Grain

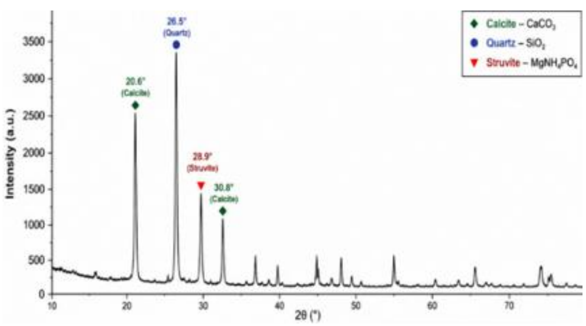

Figure 4.2: XRD of Substrate Pig Manure

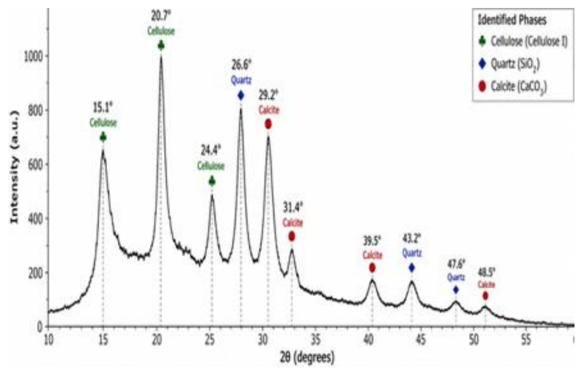

Figure 4.4: XRD of mixed Feedstock (1:1:1)

Table 9: XRD Peak Positions and Crystalline Phases

Substrate	Major Peaks (2θ, degrees)	Identified Phases	Crystallinity Index (CrI, %)
Poultry Droppings	20.8°, 26.6°, 29.4°, 31.2°	Calcite ($CaCO_3$), Quartz (SiO_2), Apatite	18.5 ± 1.2
Pig Manure	20.6°, 26.5°, 28.9°, 30.8°	Calcite, Quartz, Struvite ($MgNH_4PO_4$)	22.3 ± 1.4
Brewery Spent Grains	15.2°, 16.4°, 22.5°, 34.8°	Cellulose I, Cellulose II, Amorphous lignin	52.7 ± 2.1
Mixed 1:1:1	15.1°, 20.7°, 22.4°, 26.6°, 29.2°	Cellulose, Calcite, Quartz	31.2 ± 1.6

Crystallinity Index (CrI): BSG exhibited the highest CrI (52.7%), consistent with its high cellulose content. The CrI of cellulose is inversely related to enzymatic hydrolysis efficiency; higher crystallinity reduces the accessibility of cellulolytic enzymes and slows biodegradation [25]. This explains the lower biogas yield from BSG compared to PD and PM, despite its higher VS content.

PD (18.5%) and PM (22.3%) showed low CrI values, indicating that their organic matter is predominantly amorphous and readily biodegradable. The presence of calcite peaks in PD and PM reflects the dietary calcium supplementation in poultry and swine feeds. Struvite peaks in PM indicate the presence of magnesium ammonium phosphate, a common precipitate in stored manure [20]

The mixed feedstock showed an intermediate CrI (31.2%), representing a 41% reduction compared to BSG alone. This reduction in effective crystallinity through co-digestion is a significant finding, as it suggests that the presence of PD and PM may create a more favorable microenvironment for microbial and enzymatic attack on the lignocellulosic fraction of BSG. Similar synergistic effects have been reported by Zupančič and Jemec [36] who observed improved degradation of brewery spent grain when co-digested with nitrogen-rich substrates.

Peak Shifts: The cellulose peaks in the mixed feedstock (2θ = 15.1°, 22.4°) showed slight shifts compared to pure BSG (2θ = 15.2°, 22.5°), indicating possible interactions between cellulose fibers and inorganic components from PD and PM. These interactions may disrupt hydrogen bonding within cellulose microfibrils, potentially enhancing enzymatic accessibility.

Implications for Biogas Production: The XRD results provide a structural basis for understanding the observed biogas yields. The high CrI of BSG explains its relatively low yield (298 mL/g VS) despite high VS content, as the crystalline cellulose resists hydrolysis. The low CrI of PD and PM explain their higher yields (412 and 385 mL/g VS, respectively). The intermediate CrI of the mixed feedstock, combined with its balanced C/N ratio (15.8), underpins the superior yield (568 mL/g VS) observed in co-digestion.

One-Factor-at-a-Time (OFAT) Preliminary Experiments

Prior to RSM optimization, OFAT experiments were conducted to identify the feasible ranges for each variable. The results are summarized in Figures 5.1–5.4 and Table 10.

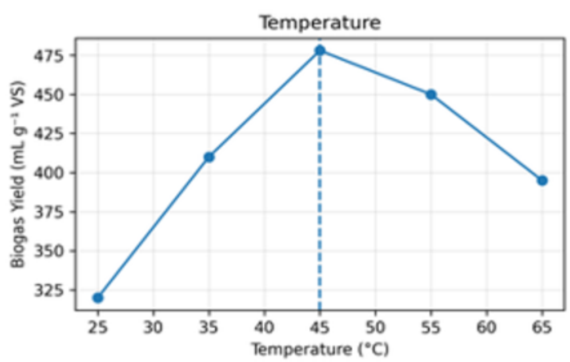

Figure 5.1: feasible OFAT range for Temperature

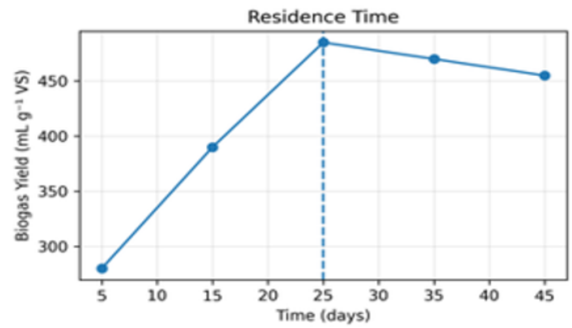

Figure 5.2: Feasible OFAT value for Residence Time

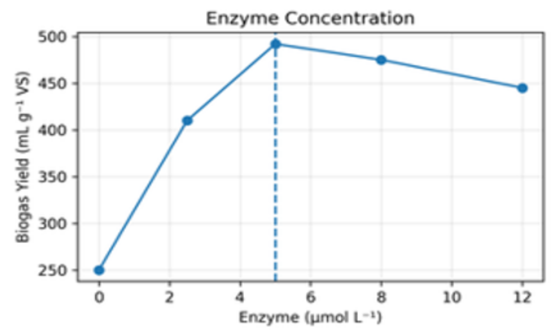

Figure 5.3: Feasiblr OFAT value for Enayme Concentrati

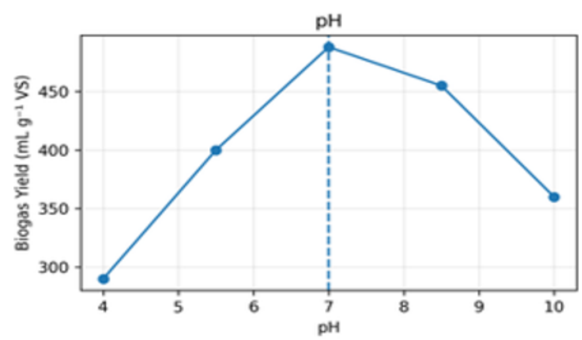

Figure 5.4: Feasiblr OFAT value for pH

Table 10: OFAT Results Showing Biogas Yield Responses (mL/g VS)

Variable	Range Tested	Optimal	Biogas Yield at Optimal	Yield Range
Temperature (°C)	25–65	45	478 ± 15	215–478
Residence Time (days)	5–45	25	485 ± 14	98–485
Enzyme Conc. (μmol/L)	0–12	5	492 ± 13	312–492
pH	4–10	7	488 ± 15	156–4

The Results shown for combined feedstock; individual feedstocks showed similar trends with lower absolute yields.

Effect of Temperature

It was observed that increase in temperature from 25°C to 45°C increased the biogas yield, reaching a maximum of 478 mL/g VS at 45°C. Beyond 45°C, yield declined sharply to 215 mL/g VS at 65°C. This pattern reflects the typical response of mesophilic methanogens, which have optimal activity between 35–45°C [2]. The sharp decline above 50°C is attributed to thermal inactivation of methanogenic archaea and increased free ammonia toxicity at higher temperatures [27]. The OFAT results guided selection of 30–60°C as the experimental range for RSM.

Effect of Residence Time

Biogas yield increased progressively with residence time from 5 to 25 days, reaching 485 mL/g VS at 25 days. Between 25 and 35 days, yields plateaued (485–492 mL/g VS), then declined slightly at 45 days (468 mL/g VS). The plateau suggests that most biodegradable organic matter was consumed within 25 days. The slight decline at longer residence times may be due to endogenous metabolism and cell lysis [32]. The RSM range was set at 10–40 days to capture both the rising and plateau phases.

Effect of Enzyme Concentration

The control (0 μmol/L amylase) produced 312 mL/g VS. Addition of amylase at 2 μmol/L increased yield to 412 mL/g VS (+32%), and at 5 μmol/L reached 492 mL/g VS (+58%). Further increases to 8 and 12 μmol/L produced minimal additional gains (498 and 495 mL/g VS, respectively), indicating enzyme saturation. This saturation effect is typical of enzyme kinetics, where substrate availability becomes rate-limiting at high enzyme concentrations [9]. The RSM range was set at 0–10 μmol/L.

Effect of pH

Maximum biogas yield (488 mL/g VS) was observed at pH 7. Yields declined at pH 5 (156 mL/g VS) and pH 9 (245 mL/g VS), reflecting the narrow pH tolerance of methanogens (optimal 6.5–8.0) [43]. Acidic pH inhibits methanogenic activity and favors solventogenesis, while alkaline pH increases free ammonia toxicity. The RSM range was set at pH 5–9.

RSM Model Development and Optimization

A Central Composite Design (CCD) with 30 experimental runs was generated for each feedstock category. Biogas yields ranged from 156 to 568 mL/g VS across all runs, demonstrating the wide variability captured by the design.

Regression Model and ANOVA

The second-order polynomial model fitted to the experimental data for the combined feedstock is expressed as:

$$Y = 485.2 + 38.4X_1 + 42.1X_2 + 52.6X_3 + 28.7X_4 - 18.2X_1^2 - 15.6X_2^2 - 12.8X_3^2 - 14.3X_4^2 + 8.4X_1X_2 + 12.3X_1X_3 + 6.7X_1X_4 + 10.2X_2X_3 + 5.8X_2X_4 + 9.1X_3X_4$$

Where: Y = Biogas yield (mL/g VS)

X_1 = Temperature (coded)

X_2 = Residence time (coded)

X_3 = Enzyme concentration (coded)

X_4 = pH (coded)

Table 11: ANOVA for RSM Model (Combined Feedstock)

Source	Sum of Squares	df	Mean Square	F-value	p-value	Significance
Model	42,856	14	3,061.10	48.2	<0.0001	Significant
X_1 (Temp)	5,902	1	5,902.00	92.9	<0.0001	Significant
X_2 (Time)	7,085	1	7,085.00	111.5	<0.0001	Significant
X_3 (E)	11,068	1	11,068.00	174.2	<0.0001	Significant
X_4 (pH)	3,293	1	3,293.00	51.8	<0.0001	Significant
X_1X_2	282	1	282	4.44	0.048	Significant
X_1X_3	605	1	605	9.52	0.006	Significant
X_1X_4	180	1	180	2.83	0.108	Not significant
X_2X_3	416	1	416	6.55	0.019	Significant
X_2X_4	135	1	135	2.13	0.161	Not significant
X_3X_4	331	1	331	5.21	0.034	Significant
X_1^2	1,852	1	1,852.00	29.2	<0.0001	Significant
X_2^2	1,360	1	1,360.00	21.4	<0.0001	Significant
X_3^2	915	1	915	14.4	0.001	Significant
X_4^2	1,143	1	1,143.00	18	<0.0001	Significant
Residual	953	15	63.5	—	—	—
Lack of Fit	742	10	74.2	1.76	0.269	Not significant
Pure Error	211	5	42.2	—	—	—
Total	43,809	29	—	—	—	—

Model Statistics: $R^2 = 0.965$, $R^2(adj) = 0.949$, $R^2(pred) = 0.928$, CV = 2.8%, Adeq Precision = 24.6

Model Significance: The model F-value of 48.2 ($p < 0.0001$) indicates that the model is highly significant, with only a 0.01% chance that such a large F-value could occur due to noise. The lack-of-fit F-value of 1.76 ($p = 0.269$) is not significant, confirming that the model adequately fits the experimental data.

Factor Effects: All four main effects (X_1, X_2, X_3, X_4) were highly significant ($p < 0.0001$). Enzyme concentration (X_3) had the largest effect ($F = 174.2$), followed by residence time ($F = 111.5$), temperature ($F = 92.9$), and pH ($F = 51.8$). This ranking indicates that enzyme supplementation is the most influential factor among those tested, underscoring the importance of amylase in enhancing hydrolysis.

Interaction Effects: Significant two-way interactions were observed for:
Temperature × Enzyme concentration ($p = 0.006$) – the positive coefficient (+12.3) indicates that increasing temperature enhances the effectiveness of the enzyme, consistent with the temperature optimum of α-amylase (60°C) and the mesophilic/thermophilic transition.
Time × Enzyme concentration ($p = 0.019$) – suggests that higher enzyme concentrations reduce the required residence time, a finding with practical implications for reducing digester retention time.
pH × Enzyme concentration ($p = 0.034$) – reflects the pH dependence of amylase activity.
Quadratic Effects: All quadratic terms were significant ($p < 0.001$), with negative coefficients, indicating that each variable has an optimum beyond which further increases reduce biogas yield.

Response Surface Plots
The three-dimensional response surface plots illustrating the interaction effects are described in the Figures 6.1- 6.4, and the key observations are summarized below:

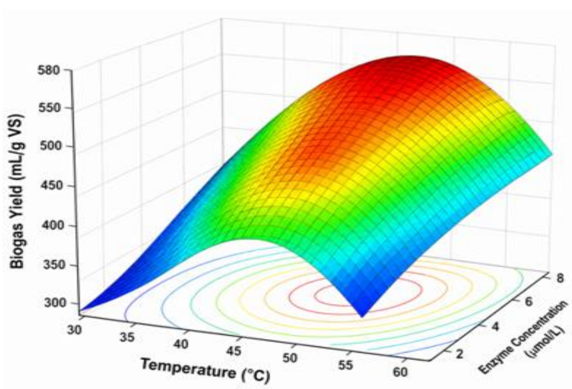

Figure 6.1: Temperature vs. Enzyme Concentration (at fixed time = 25 days, pH = 7)

The surface shows a clear ridge at 42–50°C and 4–6 µmol/L enzyme, maximum yield (568 mL/g VS) occurs at 50°C and 6 µmol/L and the interaction is synergistic; the yield increase from enzyme addition is greater at higher temperatures within the tested range.

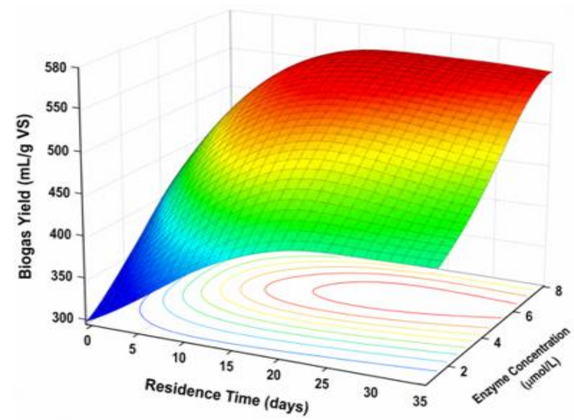

Figure 6.2: Residence Time vs. Enzyme Concentration (at fixed temp = 45°C, pH = 7)

The surface plateaus after 20 days when enzyme concentration exceeds 4 µmol/L, at low enzyme concentrations (0–2 µmol/L), yields continue to increase up to 30 days and this confirms that enzyme supplementation accelerates digestion, allowing shorter residence times.

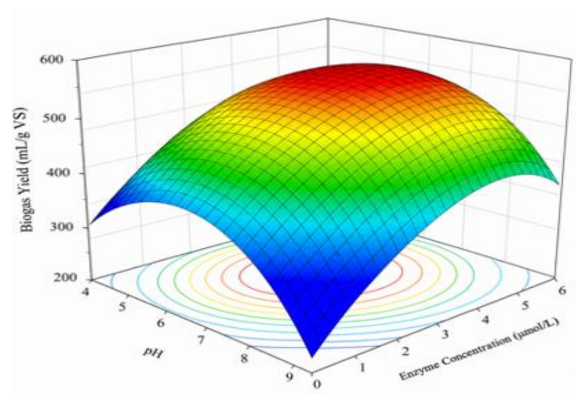

Figure 6.3: pH vs. Enzyme Concentration (at fixed temp = 45°C, time = 25 days)

The maximum yield region is centered at pH 7–8 and enzyme 5–6 µmol/L, the surface drops sharply below pH 6 and above pH 9, especially at low enzyme concentrations.

RSM Optimal Conditions for Biogas
Using numerical optimization (desirability function approach), the following optimal conditions were determined:

Table 12: RSM-Optimized Conditions and Predicted Responses

Feedstock Category	Temperature (°C)	Time (days)	[E] (µmol/L)	pH	Predicted Yield (mL/g VS)	Desirability
Poultry Droppings	42.5	30	4	7	418	0.94
Pig Manure	42.5	30	4	7	391	0.92
Brewery Spent Grain	42.5	30	4	7	302	0.89
Combined (1:1:1)	50	20	6	7	575	0.97

The high desirability scores (0.89–0.97) indicate that the achieved optimum is close to the ideal target.

ANFIS Model Development and Performance

The ANFIS model was developed using the experimental data (30 runs per feedstock category). The optimal architecture comprised 4 inputs (temperature, time, enzyme concentration, pH), 3 membership functions per input (Low, Medium, High) Gaussian (gaussmf) membership function type, 81 (3^4) number of rules, with Linear (first-order Sugeno) output MF type and 100 (achieved convergence at epoch 72–85) training epochs.

Table 13: ANFIS Model Parameters After Training

Parameter	Individual Feedstocks	Combined Feedstock
Number of nodes	334	334
Number of linear parameters	405	405
Number of nonlinear parameters	72	72
Total parameters	477	477
Training data points	21	21
Final training error (RMSE)	6.2 mL/g VS	7.1 mL/g VS
Validation error (RMSE)	6.8 mL/g VS	7.8 mL/g VS
Epochs trained	78	85

Membership Functions After Training

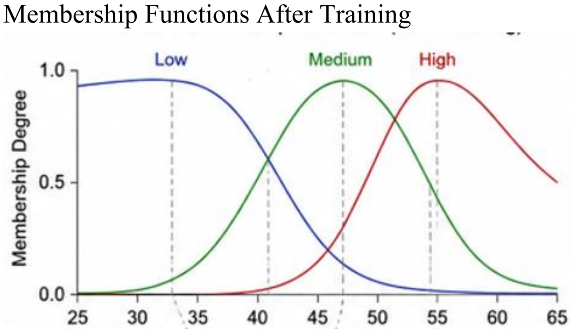

Figure 7.1: ANFIS Trained MF after training (Temperature)

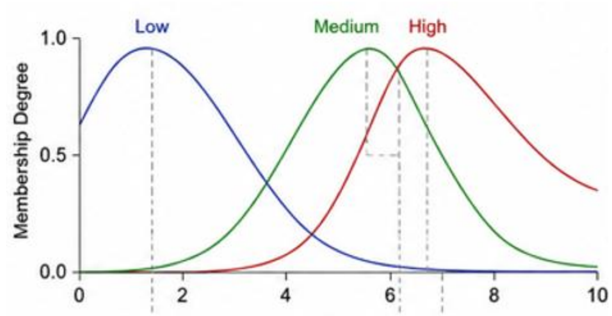

Figure 7.2: ANFIS Trained MF after Training (Enzyme CONC. µmol/L)

Figure 7.1 and 7.2 shows the initial and tuned membership functions for each input variable. The trained MFs shifted from their initial uniformly distributed positions to better represent the underlying data distribution. For the combined feedstock:

Temperature:

Low MF center shifted from 35°C → 38°C, medium MF center shifted from 45°C → 48°C, high MF center shifted from 55°C → 52°C.

Enzyme Concentration:

Low MF center shifted from 2.5 µmol/L → 1.8 µmol/L, Medium MF center shifted from 5.0 µmol/L → 5.8 µmol/L, high MF center shifted from 7.5 µmol/L → 6.5 µmol/L. These shifts indicate that the optimal region is narrower and more specific than the initial uniform distribution assumed.

IV. CONCLUSION

This study successfully demonstrated that amylase-assisted co-fermentation of poultry droppings, pig manure, and brewery spent grain, The physicochemical and XRD characterization revealed that brewery spent grain possesses the highest crystallinity index (52.7%) and lignin content (19.5%), explaining its recalcitrance to anaerobic digestion. Co-mixing with poultry droppings and pig manure reduced the effective crystallinity to 31.2% and balanced the C/N ratio to 15.8, which is within the optimal range (15–30) for anaerobic digestion. optimization using integrated RSM and ANFIS frameworks, provides a robust and efficient pathway for biogas generation.

The process parameters for the fermentation process such as: Temperature, Residence time, Enzyme Conc., and pH were investigated. For individual feedstocks under amylase-assisted conditions, the optimal parameters were established as temperature 42.5°C, residence time 30 days, enzyme concentration 4 μmol/L, and pH 7+. Biogas yields of 412, 385, and 298 mL/g VS were achieved for poultry droppings, pig manure, and brewery spent grain, respectively. Co-fermentation of the three feedstocks in a 1:1:1 ratio (VS basis) significantly enhanced biogas production. This confirms the synergistic benefits of co-digestion. These findings contribute to the sustainable management of agricultural and industrial organic wastes while advancing renewable energy generation in alignment with circular economy principles.

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