Optimization of different parameters for biogas production from mustard oil cake using BBD experimental design

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Abstract

Introduction

The increasing global energy demand and the accelerated depletion of fossil fuel reserves have prompted the urgent pursuit of sustainable and environmentally friendly energy alternatives. Biogas has emerged as a promising renewable bioenergy source due to its dual role in waste management and clean energy production. It is produced through anaerobic digestion, a microbial-driven process that breaks down biodegradable organic matter—such as agricultural residues, food waste, and wastewater sludge—under anaerobic (oxygen-free) conditions. The resulting biogas is primarily composed of methane (CH_4) ranging from 50–70%, and carbon dioxide (CO_2) constituting 30–50%, with minor fractions of other gases including hydrogen sulfide (H_2S, 0.1–1%), ammonia (NH_3), nitrogen (N_2), hydrogen (H_2), and water vapor (H_2O). The high methane content renders biogas a valuable energy carrier suitable for electricity generation, heating, and upgrading to biomethane for use as a vehicle fuel. Additionally, the anaerobic digestion process supports circular economy practices by converting organic waste into both energy and nutrient-rich digestate suitable for use as a biofertilizer **(Thirumalaivasan et al., 2024)**.

In recent years, attention has turned toward utilizing agro-industrial residues as substrates for biogas production, offering dual benefits of waste management and energy generation. The use of deoiled cake (DOC), a byproduct obtained after oil extraction from oilseeds (e.g., soybean, mustard, groundnut, cotton seed etc.) in biogas production presents several benefits due to its high organic content. Mustard oil cake (MOC), a by-product of mustard oil extraction, represents a potent substrate for biogas production owing to its high content of organic matter, including proteins and carbohydrates **(Mohanty et al., 2021)**. Traditionally used as animal feed and fertilizer, its potential for bioenergy applications remains underexploited. Efficient utilization of MOC for biogas generation aligns with the principles of a circular bioeconomy, enhancing sustainability in agriculture and energy sectors **(Ashokkumar et al., 2022)**.

However, its use is limited due to the presence of anti-nutritional compounds (ANCs) which can inhibit microbial consortia involved in anaerobic digestion, especially methanogens. As well it contain complex organic compounds (e.g., lignin, cellulose), which can slow down microbial degradation. To overcome the above limitations, DOCs often require digestion with high-moisture, enhance microbial synergy, physical, chemical, or biological pretreatment but this increases capital and operational costs. Dealing with anti-nutritional compounds (ANCs) in mustard oil cake (MOC) is essential to enhance its suitability for biogas production through anaerobic digestion. MOC contains several ANCs such as glucosinolates, phytic acid, tannins, and saponins, which can inhibit microbial activity—especially methanogens—and reduce methane yield (Sreeram et al., 2018; Chandra et al., 2012). In table we had described some of the pre treatments.

Pretreatment Methods	Method	Effect		Reference
Thermal Pretreatment	Heating MOC at 80–100°C for 30–60 minutes.	Reduces glucosinolates and denatures other inhibitory compounds.	Caution: Excessive heat can degrade volatile solids, reducing biogas potential.	Chandra et al., 2012
b) Alkaline Pretreatment	Treating MOC with NaOH, $Ca(OH)_2$, or ammonia.	Breaks down lignocellulosic structure and neutralizes tannins and saponins.	Typical dose: 2–4% NaOH (w/w) for 24 hours.	Sawatdeenarunat et al., 2015
c) Biological Pretreatment	Using fungi (e.g., Trichoderma, Aspergillus) to degrade ANCs.	Reduces phytic acid and other toxic compounds enzymatically.	Benefit: Eco-friendly and low energy input.	Gupta et al., 2016
d) Water Washing or Soaking	Soaking MOC in water for 12–24 hours and discarding the supernatant.	Leaches out water-soluble ANCs like glucosinolates and saponins.		Sreeram et al., 2018

However, the efficiency of biogas production from lignocellulosic or protein-rich substrates like MOC is significantly influenced by several process parameters such as pH, temperature, substrate concentration, carbon to nitrogen (C/N) ratio, and hydraulic retention time (HRT). Identifying and optimizing these variables is essential to enhance methane yield and process stability. Conventional methods of optimization often involve varying one parameter at a time, which fails to account for complex interactions between variables and is both time-consuming and resource-intensive **(Shahzad et al., 2024)**.

To overcome these limitations, statistical experimental design techniques such as Response Surface Methodology (RSM) have gained popularity. In particular, the Box-Behnken Design (BBD), a type of RSM, is known for its efficiency in requiring fewer experimental runs while effectively identifying optimal conditions and interactions between variables. BBD is particularly useful in modeling and optimizing bioprocesses where multiple factors simultaneously influence the response **(Veza et al., 2023)**.

In this study, we aim to optimize the key parameters affecting biogas production from mustard oil cake using BBD. By applying a systematic experimental design, we intend to develop a predictive model for methane yield and validate the model through experimental trials. This research not only contributes to the valorization of agro-industrial waste but also promotes sustainable biogas technology for decentralized energy solutions in rural and semi-urban areas.

Materials and Methods

Biogas Generation

Procurement of inoculum and substrate

The study had begun with collection of oil cakes from Various Industries. Storage of sample in air tight and cool condition. These industries uses cold-pressed technology based on screw presses for the production of oil from seeds. The oil cakes used in all experiments were from the same batch of oil production.

Fresh cattle dung (CD) was collected from Krishna Gir Dairy Farm, Visnagar. It is kept in anaerobic condition till constant methane production starts.

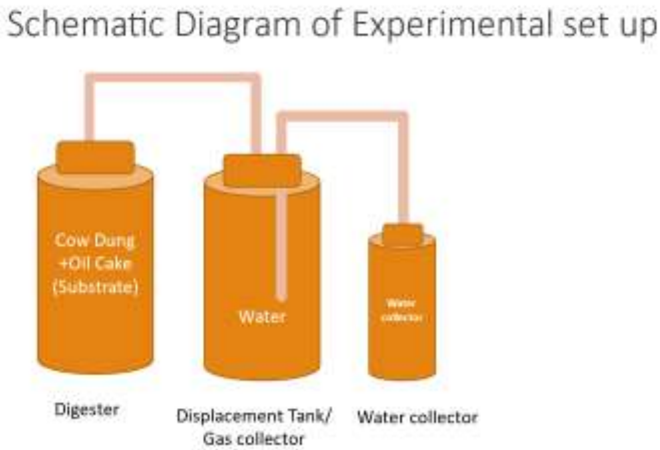
Physicochemical characterization of Inoculum and substrate

Physicochemical Characterization of sample were done as following. Characterization of sample as per CPCB and APHA. All measurements were made in triplicates, and the results are expressed as mean values. Dry matter, ash contents, moisture content, volatile solid were determined according to the standard procedures of the APHA. Estimation of celluloses was done by acetolysis followed by hydrolysis to form glucose units. These glucose units were then dehydrated and reacted with anthrone to give a green colored product, absorption of which was measured at 630 nm using a 630 nm (Özbay et al., 2001). The total carbohydrate of the oil cake was determined using the colorimetric phenol-sulfuric acid method. Nitrogen (wt.%) was used as a function to estimate the total protein of all the samples. The total lipid was estimated using the chloroform-methanol extraction method.

Anaerobic digestion

The oil cake and cow dung mixture were used as the feed. The pretreatment of the substrate makes the substrate susceptible to microbial attack and thus enhance the anaerobic digestion. Different pretreatments were checked for maximum biogas production (Table). A 2L glass bottles were used as the reactors. The experimental sets were prepared as per the directives of state ease software (table 3). To design these experiments software were given upper and lower limits of parameters which were taken with the help of references(Ref). The working volume of each reactor was 1.5L, and all experimental trials were operated at 35 ± 1 ^{0}C in a water bath. Gas will be measured by water displacement method using 2L bottle and Measuring cylinder. The schematic view of experimental setup and the photographic view of de-oiled cakes are shown in Figs. 1. Each treatment was set up in triplicate and repeated twice. Amount of oilcake and inoculum taken were designed by Design software.

Mustard oil cake



Using Stat-Ease Design expert 360 software, these variables were defined as factors under a User-defined Custom Designs section of the application. The factors were labelled A, B and C as shown in Table 3 and as obtained in the study. How the above parameters interact or relates to one another as well as the output variable were preliminarily detailed via scatter plots. The plots were created under Custom Graphs in the Navigation Pane of Stat-Ease 360 Design Expert.

User-defined Response Surface Design

In the User-defined design with both Mixture and Process factors, 3 numeric factors shown in Table 3 was specified initially. Next, the numeric factor names, units, lower and upper boundary values were entered as shown in Table 3. The response for 17 observations were as well defined accordingly. Stat-Ease 360 automatically define the type of boundary of the intervals of the parameters entered, as well as their mean and standard deviations. A quadratic model type was then chosen from the User-defined tab. Since there is only one dependent variable called response, which is the cumulative biogas yield (CBY) was specified under number of responses and the unit (ml) was entered. CBY data in Table 3 over 17 runs were then copied and pasted under Response 1 column showcased by the software. Furthermore, Quadratic was chosen as the ‘Process order’, Response type selected was ‘Design Only’ and ‘Polynomial’, as the ‘Model Type’, under Model tab under the Evaluation window.

Result and Discussion

The proximate and ultimate analysis reveals that mustard oil cake (MOC) has significantly higher carbon (58.6%) and nitrogen (2.9%) content compared to cow dung (35.2% C and 1.55% N), making it a rich organic substrate suitable for microbial conversion processes like anaerobic digestion. Its lower moisture content (11.41%) compared to cow dung (86.72%) indicates the need for hydration or co-digestion to ensure optimal microbial activity. The higher lipid content in MOC (6.92%) can contribute to increased biogas yield but may also pose a risk of long-chain fatty acid inhibition if not balanced properly; co-digestion with cow dung (0.78% lipid) can help mitigate this. Despite slightly lower volatile solids (91.49% in MOC vs. 98.73% in cow dung), MOC remains a valuable substrate, though pretreatment might improve its biodegradability due to potential lignocellulosic content (Table 1). Overall, the combination of MOC and cow dung offers a synergistic blend for efficient bioconversion, balancing nutrients, moisture, and microbial activity (**Cirne et al., 2007; Li et al., 2011).**

Table 1: Proximate and Ultimate Analysis of MOC and inoculum.

Oil Cake	**C (gm%)**	**N (gm%)**	**Moisture (gm%)**	**Total Lipid (gm%)**	**Volatile Solids (gm%)**
MOC Sample	58.6 ± 1.9	2.9 ± 0.09	11.41± 0.84	6.92 ± 0.37	91.49 ± 3.1
Cow Dung	35.2 ± 1.2	1.55± 0.04	86.72 ± 2.45	0.78 ± 0.02	98.73 ± 3.3

The data in Table 2 highlights the impact of different pretreatment methods on biogas production from mustard oil cake (MOC), showing that all treatments reached maximum biogas output at a 50% inoculum-to-substrate ratio (ISR). Among the pretreatments, hot water treatment yielded the highest cumulative biogas production (9020 ± 89 ml), indicating its superior ability to solubilize organic matter and enhance microbial accessibility, likely by disrupting lignocellulosic structures as well it may help in leaching antinutrient factors. Acid (8750 ± 79 ml) and alkali (8450 ± 74 ml) pretreatments also showed significant improvements, supporting earlier findings that chemical hydrolysis increases substrate degradability **(Chandra et al., 2007)**. Ultrasonication (7020 ± 69 ml) moderately enhanced biogas output by mechanically breaking down complex molecules, whereas cold water pretreatment resulted in the lowest yield (3510 ± 29 ml), likely due to minimal structural disruption of the substrate. These findings suggest that thermal and chemical pretreatments are more effective in enhancing biogas production from MOC than physical or low-temperature methods.

Table 2: Effect of Pretreatment on Biogas Production from MOC.

Pretreatment	**ISR at which maximum Biogas Produced**	**Cumulative Biogas Production in ml**
Acid	50%	8750 ± 79
Alkali	50%	8450 ± 74
Hot Water	50%	9020 ± 89
Cold Water	50%	3510 ± 29
Ultrasonication	50%	7020 ± 69

The results from Table 3, derived using Box-Behnken Design (BBD), illustrate the effects of three independent variables—Total Solid (TS%), Inoculum Size (%), and pH—on cumulative biogas production from mustard oil cake (MOC). The maximum biogas yield (1500 ± 26 ml) was observed in Run 11 at 9.5% TS, 20% inoculum size, and pH 6, suggesting that moderate TS levels and acidic conditions with lower inoculum concentrations favor microbial activity and substrate degradation. A similarly high yield (1400 ± 31 ml) in Run 6 (15% TS, 20% inoculum, pH 7.5) indicates that higher solids can also be effective when the inoculum remains low. Interestingly, multiple runs (5, 10, 14) at center points (9.5% TS, 55% inoculum, pH 7.5) showed consistent gas production (~1200 ml), confirming the model's reliability. However, the lowest yield (590 ± 15 ml) occurred in Run 3 (4% TS, 20% inoculum, pH 7.5), highlighting that very low solid content reduces substrate availability for digestion. Overall, the data suggest that cumulative biogas production is optimized at moderate total solids, low to mid inoculum sizes, and slightly acidic to neutral pH, aligning with previous findings that microbial efficiency is highly sensitive to substrate concentration and environmental conditions **(Mao et al., 2015; Li et al., 2011)**.

Table 3: Run (Experiment sets) given by BBD Software and Responses from MOC.

	Factor 1	Factor 2	Factor 3	Response
Run	**A: Total Solid (gm%)**	**B: Inoculum Size (%)**	**C: pH**	**Cumulative gas production in ml (MOC)**
1	15	55	6	1030 ± 22
2	9.5	90	9	900 ± 19
3	4	20	7.5	590 ± 15
4	4	55	9	850 ± 20
5	9.5	55	7.5	1200 ± 28
6	15	20	7.5	1400 ± 31
7	9.5	55	7.5	980 ± 21
8	15	55	9	1150 ± 23
9	4	55	6	1190 ± 22
10	9.5	55	7.5	1200 ± 24
11	9.5	20	6	1500 ± 26
12	9.5	55	7.5	980 ± 13
13	9.5	20	6	820 ± 11
14	9.5	55	7.5	1190 ± 15
15	4	90	7.5	1366 ± 14
16	9.5	20	9	1020 ± 10
17	15	90	7.5	840 ± 13

ANOVA for Quadratic Model

The ANOVA results for the quadratic model evaluating MOC (Mustard Oil Cake) indicate that the model is statistically significant ($F = 15.09$, $p = 0.0002$), demonstrating a good fit for the data. Among the individual factors, inoculum percentage (B) and pH (C) significantly influenced MOC with p-values of 0.0156 and 0.0424, respectively, while solid concentration (A) showed no significant effect ($p = 0.1429$). Importantly, the interaction effects AB (solid concentration × inoculum percent), AC (solid concentration × pH), and BC (inoculum percent × pH) were all significant, with AB showing the strongest interaction ($F = 50.2$, $p < 0.0001$), suggesting that the combined effects of these variables are more influential than their individual contributions. Furthermore, the non-significant lack of fit ($F = 0.384$, $p = 0.8583$) confirms the adequacy of the model, indicating that the variability in the data is well-explained by the regression **(Table 4)**. These results align with previous studies emphasizing the synergistic effects of fermentation parameters in optimizing microbial processes **(Montgomery, 2017; Bezerra et al., 2008)**.

The coefficient of variation (COV), sometimes referred to as relative standard deviation (*SD*), is the percentage of the ratio between *SD* and the mean (Stat-Ease Inc., 2021). The COV percentage is used to judge the capability of a process; lower is better as it suggest more accurate models. The COV percentage of the biogas yield model was 8.80%, which is very low. Therefore, the standard deviation (94.28) is very low in comparison to the mean (1070.94), which suggest that the biogas yield model is accurate.

Adeq Precision measures the signal to noise ratio. Adequate Precision (*AP*) compares the limits of the predicted observational response to the average estimate error/noise i.e. a signal to noise ratio (Stat-Ease Inc., 2019). Higher values indicate a very high range in the predicted response compared to its associated error. As a result, an *AP*ratio that is greater than 4.0 implies that the model discrimination is satisfactory (Stat-Ease Inc., 2019). From the table, the *AP*ratio for biogas yield was 14.249, which is greater than 4.0 and thus indicating satisfactory model. Thus, the chosen model may be applied for navigating the design space since the estimated signal is lesser affected by noise (Stat-Ease Inc., 2019 Frost, 2021;).

The fit model statistics for MOC (Table 5) further validate the robustness and predictive ability of the quadratic model. A high R^2 value of 0.90 indicates that 90% of the variability in MOC is explained by the model, while the adjusted R^2 (0.84) and predicted R^2 (0.78) are reasonably close, reflecting a good balance between model complexity and predictive accuracy. The coefficient of variation (C.V.) at 8.8% signifies acceptable precision and reliability of the experimental data, as values below 10% are generally considered desirable in biological experiments **(Montgomery, 2017)**. The Adequate Precision value of 14.25, which is well above the threshold value of 4, suggests a strong signal-to-noise ratio, further confirming that the model can be used to navigate the design space effectively. Overall, these statistics support the use of the model for reliable prediction and optimization of MOC under the tested conditions.

Table 4: ANOVA for Quadratic model for MOC.

Source	**Sum of Squares**	**df**	**Mean Square**	**F-value**	**p-value**	
Model	**8.049E+05**	**6**	**1.342E+05**	**15.09**	**0.0002**	**significant**
A-Solid concentration	**22472.00**	**1**	**22472.00**	**2.53**	**0.1429**	
B-Inoculum percent	**75272.00**	**1**	**75272.00**	**8.47**	**0.0156**	
C-pH	**48050.00**	**1**	**48050.00**	**5.41**	**0.0424**	
AB	**4.462E+05**	**1**	**4.462E+05**	**50.20**	**< 0.0001**	
AC	**52900.00**	**1**	**52900.00**	**5.95**	**0.0349**	

BC	**1.600E+05**	**1**	**1.600E+05**	**18.00**	**0.0017**	
Residual	**88882.94**	**10**	**8888.29**			
Lack of Fit	**32482.94**	**6**	**5413.82**	**0.3840**	**0.8583**	**not significant**
Pure Error	**56400.00**	**4**	**14100.00**			
Cor Total	**8.938E+05**	**16**				

Table 5: Fit Model Statistics for MOC.

Std. Dev.	94.28	**R^2**	0.90
Mean	1070.9	**Adjusted R^2**	0.84
C.V. %	8.8	**Predicted R^2**	0.78
		Adeq Precision	14.25

Two Variables Response Contour Plots

3D surface and contour plots useful for investigating desirable response values and operating conditions, contains predictors on the x- and y-axes and continuous surface that represents the response values on the z-axis. The peaks and valleys as shown in Figures 3-12 (contor graphs), correspond with combinations of x and y that produce local maxima or minima. A 3D surface plot curved upward in Stat-Ease typically indicates that the response variable increases as both predictor variables increase. This suggests a positive relationship between the variables. To determine the points corresponding to the local minimum along the x and y axes, analysis of the contour lines on the surface plot was carried out.

Although counter plots are very useful in statistics, they only display two-dimensional dimension (2D) of the variables, which makes it not easy to obtain the correct optimum value of a function. A very useful plot that makes it easier to see the true representation of a function is a three-dimensional surface (3D). Fig. 3**a**, Fig. 3**b**, and Fig. 3**c** show 3D diagrams for inoculum percent and solid concentration, pH and inoculum percent, pH and solid concentration interactions for biogas yield, respectively.

From the plots, the optimum biogas yield is approximately 2833ml and was achieved at a solid concentration 12.93, Inoculum percent 27.28 and pH 7.7.

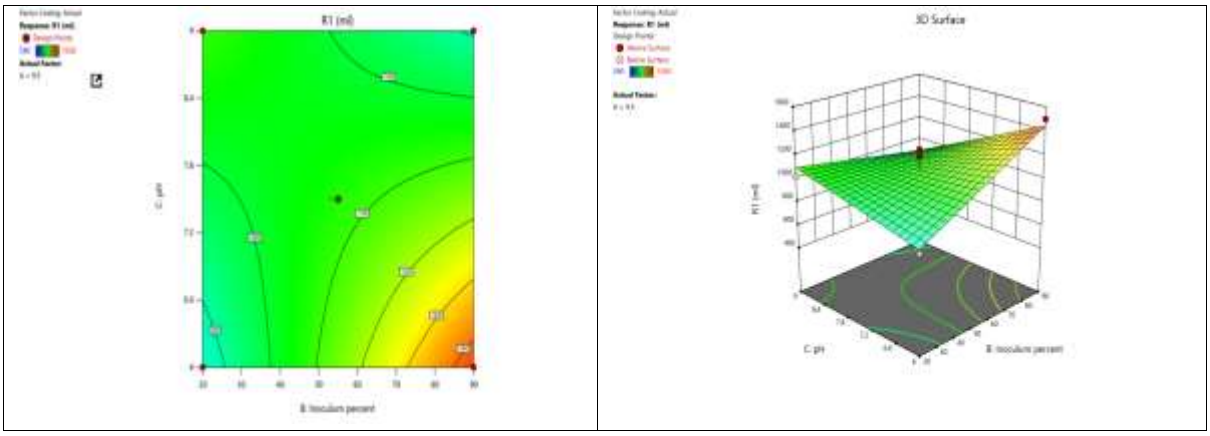


Figure 1: 2 D Graph & 3D graph for Total Biogas Production from MOC showing comparison of pH and Inoculum Percent.

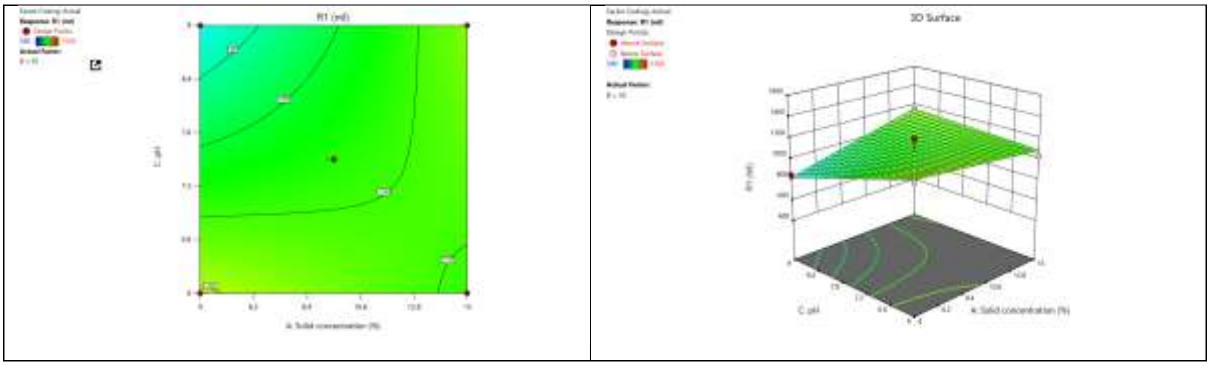


Figure 2: 2 D Graph & 3D graph for Total Biogas Production from MOC showing comparison of Solid Concentration and pH.

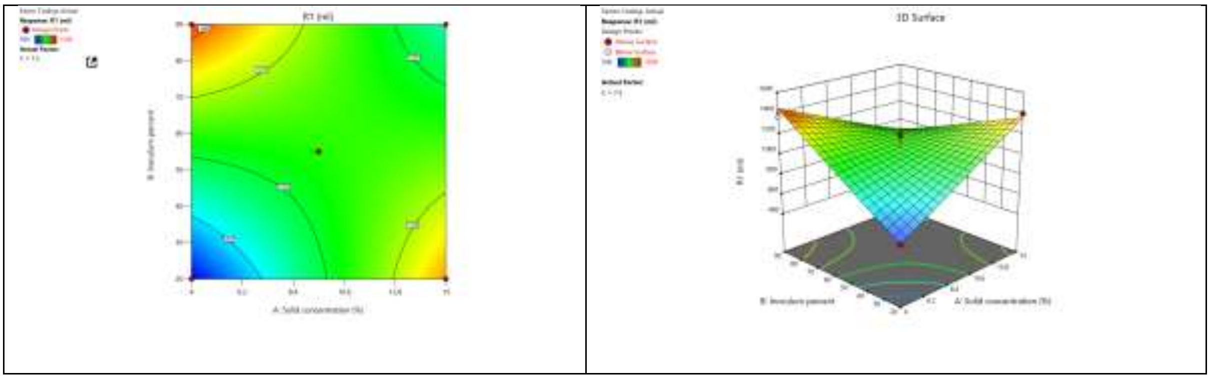


Figure 3: 2 D Graph & 3D graph for Total Biogas Production from MOC showing comparison of Solid Concentration and Inoculum Percent.

The comparison between predicted and experimental values for MOC (Table 6) demonstrates the model's strong predictive capability, with the predicted value of 1502 being close to the experimental value of 1680 under the same conditions (solid concentration: 15%, inoculum percent: 20%, pH: ~8). Although a slight deviation exists, such differences are expected in biological systems due to inherent variability and operational fluctuations. The close agreement supports the validity of the quadratic model developed, indicating that it can reliably forecast outcomes within the tested design space. Similar findings have been reported in optimization studies where predicted values closely matched experimental data, affirming the effectiveness of response surface methodology **(Bezerra et al., 2008; Montgomery, 2017)**.

Table 6: Predicted verses experimental value.

Oil Cake	Number	Solid concentration	Inoculum percent	pH	R1
MOC	**Predicted**	15	20	8.39	1502
	Experimental	15	20	8	1680

Conclusion

The aforementioned studies demonstrate that the application of software-based optimization techniques can significantly enhance biogas production from oil cake substrates. Through computational modeling and statistical design of experiments, key operational parameters such as substrate concentration, inoculum ratio, temperature, pH, and hydraulic retention time can be optimized efficiently. This systematic approach enables the identification of the most influential factors affecting biogas yield and provides an accurate prediction of process outcomes. Moreover, software-assisted optimization minimizes the need for extensive experimental trials, thereby saving time, reducing costs, and improving process reproducibility. The result shows that the biogas production in experimental analysis (1680 mL) is more than that of predicted value (1502 ml). Hence, integrating software-based optimization in biogas production studies represents a reliable and sustainable strategy for maximizing energy recovery from oil cake and other agro-industrial wastes.

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