Optimization of the thermophilic anaerobic co-digestion of pig manure, agriculture waste and inorganic additive through specific methanogenic activity

J. Jiménez, M. E. Cisneros-Ortiz, Y. Guardia-Puebla, J. M. Morgan-Sagastume and A. Noyola

ABSTRACT

The anaerobic co-digestion of three wastes (manure, rice straw and clay residue, an inorganic additive) at different concentration levels and their interactive effects on methanogenic activity were investigated in this work at thermophilic conditions in order to enhance hydrolytic activity and methane production. A central composite design and the response surface methodology were applied for the optimization of specific methanogenic activity (SMA) by assessing their interaction effects with a reduced number of experiments. The results showed a significant interaction among the wastes on the SMA and confirmed that co-digestion enhances methane production. Rice straw apparently did not supply a significant amount of substrate to make a difference in SMA or methane yield. On the other hand, clay residue had a positive effect as an inorganic additive for stimulating the anaerobic process, based on its mineral content and its adsorbent properties for ammonia. Finally, the optimal conditions for achieving a thermophilic SMA value close to 1.4 g CH4-COD/g VSS · d−1 were 20.3 gVSS/L of manure, 9.8 gVSS/L of rice straw and 3.3 gTSS/L of clay.

Key words | methanogenic activity, optimization, response surface methodology, thermophilic co-digestion

INTRODUCTION

The anaerobic digestion of manure could be enhanced using organic agriculture wastes as co-substrate, due to improvement of the C/N balance (Campos 2001). Agriculture wastes such as rice straw could be a promising feedstock biomass as co-substrate for pig manure anaerobic digestion, due to their availability and low cost (Wang et al. 2009) and their high carbon and other nutrients content. However, rice straw contains lignocellulose compounds that would limit its anaerobic biodegradation (Mussatto et al. 2008). In order to partly overcome this limitation, a thermophilic anaerobic process may be applied, since at this operational temperature the digestibility of straw increases (Zhang & Zhang 1999).

On the other hand, other factors contribute to hinder methane production from manures, namely high ammonia concentration, which may be inhibitory to anaerobic digestion (Sung & Liu 2003; Rajagopal et al. 2015). To solve this problem, several inorganic additives, such as heavy metals, zeolites and clay can improve the drawback of process instability caused by ammonia inhibition (Angelidaki & Ahring 1993; Hansen et al. 1998; Montalvo et al. 2005). This is of great importance because methanogens are the microorganisms least tolerant to high ammonia concentration (Kayhian 1994; Karakashev et al. 2005; Nettmann et al. 2010). In this sense, the anaerobic digestion of swine manure (ammonia rich substrate) has been improved using clay and zeolite as inorganic additives, e.g. glauconite, natural zeolite (clinoptilolite, mordenite) and montmorillonite (Hansen et al. 1999; Milán et al. 2001; Duran-Barrantes et al. 2008; Kotsopoulos et al. 2008; Rajagopal et al. 2015).

Many environmental factors also affect methane yield, including substrate concentration, temperature, etc. In this multi-component system, response surface methodology
can be proposed in order to determine the influence of individual wastes and their interactive effects on the specific methanogenic activity (SMA), taking this parameter as a measure of methane yield and methanogen behavior. The response surface methodology is a statistical technique for designing experiments, building models, evaluating the effects of several factors and searching optimum conditions for desirable responses, based on a limited number of experiments. Additionally, a central composite design is a factorial design effective for sequential experimentation and allows a reasonable amount of information for testing lack of fit (Montgomery 2005).

The interactive effects of concentration levels on the methane production process from co-digestion of manure, rice straw and clay residues as an inorganic additive have not been reported yet. Consequently, the objective of this work was to investigate the effect of different concentrations of these solid wastes, as well as their interactions, on SMA in thermophilic anaerobic conditions, using a central composite design and the response surface methodology.

METHODS

The inoculum and wastes

Anaerobic sludge fed with pig manure and adapted to thermophilic conditions (55 ± 2 °C) was used as inoculum. The wastes analyzed were pig manure (solid excreta), rice straw as agriculture waste and clay residual from the oil clarification process as an inorganic additive. The pig manure was collected in the Veterinary School, UNAM. Rice straw and clay residual were collected from two Cuban enterprises and they were kept at room temperature until used.

Rice straw (crop residue of the rice plant) was collected randomly in rice fields of Rice Company ‘Sur del Jibaro’ Sancti Spiritus, Cuba. This was subsequently strongly crushed (crusher Severin, Gmb, Germany) and sieved through meshes of 2 mm. Clay waste obtained from purification processes of industrial base oils, were kindly donated by environmental managers of the Petrochemical Industry, Oil Refinery ‘Sergio Soto’ in Sancti Spiritus, Cuba.

Optimization studies

A full 2³ factorial central composite design and the response surface methodology were used supported by the Statgraphics software (Version 5.1). The design was improved by six axial points, and six replications of center points. Sixteen different mixtures were assayed (Table 1). The batch experiments were carried out in 60 mL bottles containing 3.8 g volatile suspended solids (VSS)/L of inoculum and growth media. All bottles were inoculated and prepared in an anaerobic chamber and then incubated at thermophilic conditions (55 ± 2 °C) for 25 days. Blanks used were the inoculum without substrate (B₀), and the inoculum with pig manure (B₀+M). Each mixture was run in triplicate. The wastes were chosen as the three independent variables and SMA was the dependent (response) variable.

The SMA variable was fitted using a predictive polynomial quadratic equation in order to correlate it with the independent variables. The quality of fit of the model equation was expressed by the determination coefficient \( R^2 \), and its statistical significance was determined by an F-test based on the p-value with 95% confidence level. The significance of the regression model was tested by a t-test. The standardized effects of the independent variables and their interactions on the dependent variable were also evaluated with a Pareto chart. The Durbin–Watson statistic was used to determine if autocorrelation of first order existed. Finally, three-dimensional (3D) response surfaces and two-dimensional contour plots were constructed to give an insight about the effects of different wastes and their interaction on SMA.

Table 1 | Waste concentration level used for each mixture tested

<table>
<thead>
<tr>
<th>Wastes</th>
<th>Batch test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6  7  8  9  10  11  12  13  14  15  16</td>
</tr>
<tr>
<td>Manurea</td>
<td>22.85 9.1 36.6 9.1 36.6 9.1 36.6 9.1 36.6 9.1 36.6 22.85 22.85 22.85 22.85</td>
</tr>
<tr>
<td>Strawa</td>
<td>12.3 7.0 7.0 17.6 17.6 7.0 7.0 17.6 17.6 12.3 12.3 7.0 17.6 12.3 12.3</td>
</tr>
<tr>
<td>Clayb</td>
<td>4.55 0.8 0.8 0.8 0.8 8.3 8.3 8.3 8.3 4.55 4.55 4.55 4.55 0.8 8.3 4.55</td>
</tr>
</tbody>
</table>

\( ^a \) gVSS/L.
\( ^b \) gTSS/L.
Determination of the adsorption capacity of clay

To determine the adsorptive properties of clay residual, the adsorption coefficient was determined using the Freundlich isotherm according to the method proposed by Wang et al. (2011), with the modification of using different concentrations of the clay. Thus 20, 50, 93 and 172 g/L of clay were added to 0.04 L of ammonium chloride in a concentration range between 3 and 7 g/L. The tubes were maintained in a water bath at 37 °C for 24 h. Subsequently, the concentration of ammonium ions remaining in the solution was analyzed by distillation method (Vapodest, Gerhardt, Germany). Controls for each ammonium concentration were used without clay addition. The amount of adsorbed ammonium ions was calculated according to the equation proposed by Wang et al. (2011)

\[
G = \frac{(C_0 - C_{eq})}{m} V
\]

where \( G \) is the amount of ammonia adsorbed per unit mass of clay (mg/g). \( C_0 \) is the concentration of ammonium of control (initial) for each concentration tested (mg/L). \( C_{eq} \) is the equilibrium concentration at the time of measurement (obtained final concentration, mg/L). \( V \) is the total volume of solution and \( m \) is the mass of clay added in each case.

Subsequently, the model was used to determine Freundlich adsorption coefficients of the material. These coefficients were determined by the mathematical function

\[
G = K_f C_{eq}^{1/n}
\]

where \( K_f \) is the adsorption capacity or Freundlich adsorption coefficients and \( n \) is the intensity of the adsorption isotherm. The resulting linear equation is obtained by applying the logarithmic function, where it can extrapolate the experimental data

\[
\log G = \log K_f + \frac{1}{n} \log C_{eq}
\]

Analytical techniques

Several physico-chemical parameters were determined for monitoring the anaerobic process: total suspended solids (TSS), VSS, pH and alkalinity were determined according to Standard Methods for the Examination of Water and Wastewater (APHA 1995). The alkalinity ratio \((\alpha)\) was calculated as the quotient of partial alkalinity (at pH 5.75) and total alkalinity (at pH 4.3). Methane production was determined every day by gas chromatography (Fisher gas partitioner model 1,200 with a thermal conductivity detector and a Porapak Q column). Subsequently, the total methane in the bottle gas space was determined.

Specific methanogenic Activity

SMA was calculated with the slope of the accumulated methane production curve (mole·d⁻¹) in the first 5 to 6 days, divided by the amount of VSS introduced in the bottle (inoculum) using the proper conversion factor to report it as gCH₄·COD·gVSS⁻¹·d⁻¹ (COD: chemical oxygen demand).

RESULTS AND DISCUSSION

Designing mixture combinations, using the response surface methodology allowed the assessment of a wide range of concentrations of the three substrates at a time, in a limited number of experiments (Montgomery 2005).

The profile of methane production during the co-digestion of three wastes at different concentration levels showed different responses (Figure 1). An increase in methane production was achieved for almost every mixture with respect to the blanks, similar to the results obtained by Moller et al. (2004), Wang et al. (2009) and Mussoline et al. (2012). Mixture 6 (higher concentration of clay with lower levels of manure and straw) showed an inhibitory effect partially due to the fact that this inorganic additive is not an organic substrate, so the low methane production was obtained just from the manure and straw.
The higher cumulative methane production was accomplished with mixtures 14 and 11, followed by mixtures 12 and 3. Both groups had a high and a medium level mixture of manure, with the medium level of rice straw for the first group and the low level for the second group. In addition, the physico-chemical values obtained at the end of each mixture batch test remained in the following intervals: pH (7.4–8.1), alkalinity (2.8–3.8 gCaCO₃/L), α (0.6–0.8) and the VSS removal (65–85%), indicating proper conditions for anaerobic digestion in all cases (Mussoline et al. 2012).

The statistical evaluation, the regression model, effects of model components and their interactions on SMA

The SMA results were fitted to a second-order polynomial equation using a multiple regression analysis. Consequently, the mathematical regression model for SMA fitted in terms of uncoded factors was obtained as follows:

\[
SMA = 0.358717 + 0.0899109 \cdot A + 0.01027 \cdot B - 0.0793558 \cdot C - 0.00290271 \cdot A^2 + 0.00100343 \cdot A \cdot B \\
+ 0.0056667 \cdot A \cdot C - 0.00369502 \cdot B^2 \\
+ 0.00091195 \cdot B \cdot C - 0.0061364 \cdot C^2
\]

where \( A \) is manure, \( B \) is rice straw and \( C \) is clay (concentration units as reported in Table 1).

The SMA values of anaerobic digestion systems of different combinations of mixture showed a wide range. The results indicate that the SMA was strongly affected by the substrates, although for both temperature conditions, no apparent effect of the straw was observed, probably because of its complex lignocellulosic structure, which limits the hydrolytic step of the process and therefore retards the methanogenesis (Zhang & Zhang 1999; Mussatto et al. 2008). Including straw showed no effect on therophilic conditions, where it is assumed that high temperatures favor hydrolysis (Zhang & Zhang 1999). This might be due to the batch fermentation time (21 days) not being sufficient for complete hydrolysis of lignocellulosic material, which prevents the bioavailability of carbon content in rice straw.

The analysis of variance (ANOVA) showed that manure (F-ratio = 18.26; p-value = 0.0005) and straw (F-ratio = 19.23; p-value = 0.0004) had a significant effect on SMA (95% confidence level; p-value <0.05). The interaction between manure and straw (F-ratio = 13.52; p-value = 0.0019) and the interaction between manure and clay (F-ratio = 200.94; p-value = 0.0000) were also significant on SMA. However the clay levels and the interaction between straw and clay had no significant effect on the response SMA. In addition, the quadratic component of manure (F-ratio = 251.01; p-value = 0.0000) and rice straw (F-ratio = 8.98; p-value = 0.0081) showed a significant effect on SMA.

The quality of fit of the model equation expressed as the determination coefficient (\( R^2 = 0.9612 \)) showed that only 3.89% of total variation was not explained by the regression model. Also, the adjusted determination coefficient (Adj. \( R^2 = 0.9453 \)) showed the model adequacy. The Durbin–Watson statistic close to 2 (Durbin–Watson = 2.4761) indicated that there was no correlation among the residues (error-true estimation) and thus confirmed the fitness of the regression model. In general, this descriptive statistics showed the goodness of fit of the models at the temperature assayed, similar to those achieved by other authors who have used the response surface methodology (Liu et al. 2004; Yetilmезsöy et al. 2009). This contributes to the search for methods about methanogenesis modeling from different types of substrates.

The standardized effects of the independent variables and their interactions on the dependent variable were also determined by a Pareto chart (Figure 2). This approach identifies the factors that affect at least 80% of SMA values.

The standardized effect of clay and the interaction between straw and clay were higher than the reference line, so they had a positive (synergetic) effect on SMA, similar to the results of Campos (2001) during the co-digestion of manure with agricultural waste and clay from olive oil clarification. On the other hand, the negative coefficients higher
than the reference value obtained for manure, its quadratic component, the interaction manure–rice straw, manure–clay and the quadratic component of rice straw showed an antagonistic effect on SMA. The standarized effect of rice straw and the quadratic component of clay remained under the reference line, with no effect on SMA. Apparently, even at thermophilic conditions, straw (a lignocellulosic material) does not represent an available substrate to methanogenesis consortia, due to its low rate of hydrolysis (Zhang & Zhang 1999; Mussatto et al. 2008; Mussoline et al. 2013).

### 3D response surface for SMA

The 3D response surface plots are a function of two independent variables, maintaining others at fixed levels. They are represented as a solid surface in a 3D space. Figure 3 shows the 3D response surface plots for SMA in three sets: rice straw–clay with fixed levels of manure; manure–clay with fixed levels of rice straw and manure–rice straw with fixed levels of clay. The graphical representation of the response surface supported the effect of the main factors and their interaction on the SMA (Adinarayana & Ellaiah 2014).
2002; Yetilmezsoy et al. 2009). Thus, the nonlinear nature of all surfaces of response obtained and the respective contours, showed that there is considerable interaction between the three substrates evaluated.

For the first set, the higher SMA values (1.14–1.45 gCH₄·COD/gVSS·d⁻¹) were obtained at the central level of manure (22.85 gVSS/L). At the lower level (9.1 gVSS/L) clay concentration had a detrimental effect on SMA, while at its higher level (36.6 gVSS/L) a clear inhibitory effect was reduced by increasing clay content. This result could be explained taking into account the partial inhibition of SMA due to the concentration of ammonia nitrogen in manure.

Ammonia can be absorbed by clay residual assayed. The adsorbent properties of clay residual were assessed in parallel batch experiments according to the isotherm Freundlich by contacting different concentrations of clays with known concentrations of ammonium ions in solution. The results show the coefficients obtained for each concentration of clay residual used, with adjustments to the model, determined by the coefficient of determination ($R^2$) (Table 2).

The better performance of clay as adsorbent, shown for concentrations between 50 and 93 g/L of fresh matter of clay added, where the best fit was obtained with the model described by equation or Freundlich isotherm with adsorption coefficients, $K_f = 1.43$ and $n_f = 1.82$, respectively. Furthermore, the intensities of the isotherm in both cases, $n$ (1.29 and 2.98, respectively) show the strength of adsorbent–adsorbed binding. However, it is observed that there is lack of fit of the model when low concentrations of clay (20 g/L of clay added, $R^2 = 0.87$) or very high concentrations of this are used (172 g/L of fresh matter added clay, $R^2 = 0.70$). These results are similar to those obtained by Hansen et al. (1999), Milán et al. (2003) and Tada et al. (2005) using similar compounds, such as bentonite, natural zeolite like mordenite and others.

The 3D response surface plot for the second set showed a similar pattern for the three levels of straw, leading to the conclusion that rice straw had no real effect on SMA. This result may be due to the low hydrolysis rate of this compound, considering its lignocellulosic content (Zhang & Zhang 1999; Mussatto et al. 2008). The inhibitory effect at higher manure content is again evident, with a positive effect of clay, as mentioned previously.

Finally, the 3D response surface plot for the third set showed a very limited effect of rice straw on SMA at the three levels of clay content. Also, these results clearly confirm the inhibitory effect of manure at concentrations higher than 25 gVSS/L and the role of clay as a helpful additive. As mentioned, the sorption capability of clay may explain this positive behaviour.

**Optimization studies for maximizing the production of SMA**

The second-order polynomial model applied in this study was used for determining the optimum conditions in order to maximize SMA. The ANOVA analysis showed that the quadratic model was adequate for data observed at 95% confidence level, while the regression analysis was highly significant, therefore it was concluded that the second-order polynomial model represented closely the actual response surface. The optimal conditions for a SMA of 1.38 gCH₄·COD/gVSS·d⁻¹ were determined as 20.29 gVSS/L of manure, 9.80 gVSS/L of rice straw and 3.30 gTSS/L of clay (Figure 4).

The main factor that influenced the SMA was the concentration of pig manure because manure contains high concentrations of ammonia nitrogen concentrations, which may be inhibitory to the microbial community associated with the production of methane, as has been reported by several authors (Hansen et al. 1998; Campos 2001; Sung & Liu 2005). Given this phenomenon, the positive effect of the residual clay and ammonium ion adsorbent was evident, which counteracts its inhibitory effect, similar to that obtained by others using natural and modified zeolites and other clay materials (Angelidaki & Ahring 1993; Hansen et al. 1999; Milán et al. 2003; Tada et al. 2005).

The maximized SMA obtained at the experimental thermophilic condition used in this work may be considered as a representative value for this kind of waste (Soto et al. 1993; Rifat & Krongthamchat 2007; Laubie et al. 2010). Also, the statistical analysis and data handling used in this study proved to be useful for optimizing operational conditions in order to enhance methanogenic activity and methane yield from co-digestion of organic wastes. In general, the values of SMA anaerobic sludge reactors that degrade organic waste as manure and agricultural waste nature are much lower, ranging in rank between 0.1 and 1.0 gCH₄·DOQ/gSSV·L⁻¹. From this, the importance of this work

**Table 2** | Freundlich coefficient ($K_f$) and adsorption capacity ($n$) of clay residual used. Each value is an average of three replicates ± standard deviation

<table>
<thead>
<tr>
<th>Clay residual concentration (g/L)</th>
<th>$K_f$</th>
<th>$n$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1.12 ± 0.1</td>
<td>0.67 ± 0.1</td>
<td>0.87</td>
</tr>
<tr>
<td>50</td>
<td>1.43 ± 0.0</td>
<td>1.29 ± 0.0</td>
<td>0.99</td>
</tr>
<tr>
<td>93</td>
<td>1.82 ± 0.0</td>
<td>2.98 ± 0.0</td>
<td>0.96</td>
</tr>
<tr>
<td>172</td>
<td>0.75 ± 0.1</td>
<td>1.79 ± 0.1</td>
<td>0.70</td>
</tr>
</tbody>
</table>
to optimize the SMA during anaerobic co-digestion of organic waste is derived, as a key step in the metabolic pathway of methane formation. This further confirms that the central composite design, the statistical model and response surface methodology are powerful tools for the optimization of parameters in these systems of anaerobic co-digestion of waste of different nature (Bhunia & Ghangrekar 2008; Wang & Wan 2008).

CONCLUSIONS

The optimization of SMA for anaerobic co-digestion of pig manure, rice straw and clay residue was accomplished using the central composite experimental design and the 3D response surface methodology.

The statistical analysis of the SMA results showed a significant interaction between manure and the two other wastes. However, rice straw was not available for methanogenesis, resulting in no real effect on SMA. Apparently, the thermophilic condition did not increase its hydrolysis rate.

Manure presented an inhibitory behaviour at concentrations higher than 25 gVSS/L but the clay additive reduced the detrimental effect on SMA. The adsorption capacity of clay, which avoids the ammonia excess, demonstrated in this study, may explain this result.

The optimal conditions in order to obtain a thermophilic SMA close to 1.4 gCH₄-COD/VSS · d⁻¹ are the following: 20.3 gVSS/L of manure, 9.8 gVSS/L of rice straw and 3.3 gTSS/L of clay.

The design and the optimization of culture media for the methane obtaining, by the anaerobic digestion of pig manure, straw of rice and clay residuals, allowed us to elevate the SMA to values higher than those informed when pig manure is used as mono-substrate. With this culture media, high methane yields are achieved, using higher organic loads of manure, without the methanogen inhibition caused by ammonia nitrogen.

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reactors with clays supports. *Chemical and Biochemical Engineering Quarterly* 22, 393–399.


