Characterization of a Lignocellulolytic Consortium and Methane Production from Untreated Wheat Straw: Dependence on Nitrogen and Phosphorous Content

Idania Valdez-Vazquez,[a,*] Gabriela J. Torres-Aguirre,[b] Carlos Molina,[c] and Graciela M. L. Ruiz-Aguilar [b]

Impacts of microbial diversity and macronutrients levels (expressed as C:N and C:P ratios) on the methane production from an untreated lignocellulosic feedstock were assessed. Next-generation sequencing technology revealed the bacterial diversity of a lignocellulolytic inoculum. This inoculum comprised 75 bacterial species that were well distributed in 14 phyla, 67% of which belonged to *Firmicutes* and *Bacteroidetes*. The families Ruminococcaceae, Clostridiaceae, Bacteroidaceae, Bacillaceae, and Fibrobacteraceae comprised 46% of the identified families and were associated with hydrolytic members. Nutrient adjustment reduced 40% of the length of the lag phase and doubled methane production rate compared with a control. The highest methane production of 0.197 m^3 per kg of total volatile solids observed at C:N of 31:1 and C:P of 428:1, peaked 20 days earlier than in previous studies using untreated lignocellulosic feedstock. Interestingly, the highest hydrolytic activities and solids removal rates were observed at high nitrogen contents; however, the conditions (pH > 8.0) inhibited methanogenesis.

Keywords: Anaerobic digestion; Consortium; Pyrosequencing; Winter wheat (Triticum aestivum L.)

*Contact information: a: Unidad Académica Juriquilla, Instituto de Ingeniería, Universidad Nacional Autónoma de México, Blvd. Juriquilla 3001, 76230 Querétaro, Mexico; b: División de Ciencias de la Vida, Universidad de Guanajuato, Irapuato, Mexico; c: División de Ciencias e Ingenierías, Universidad de Guanajuato, León, Mexico; *Corresponding author: IValdezV@iingen.unam.mx*

INTRODUCTION

Anaerobic digestion (AD) transforms diverse organic wastes into a methane-rich gas, which is used worldwide for generating power. Anaerobic digestion is the most efficient technology compared with other means of energy production through biological or thermochemical routes (Gerardi 2003; Deublein and Steinhauser 2008). Thus, there are thousands of full-scale digesters worldwide, most of which are fed with industrial wastewater, food waste, animal manure, and sewage sludge (Sawatdeenarunat *et al*. 2015).

Recently, there has been renewed interest in the anaerobic digestion of lignocellulosic biomass (LB), primarily because of the urgent need for bioenergy to satisfy growing energy demands, to reduce the use of non-renewable fossil fuels, and to decrease CO_2 emissions. Lignocellulosic biomass is a primary candidate for generating bioenergy due to its abundance and renewability (Chandra *et al*. 2012). In Mexico, crop wastes are the most abundantly available LB (Rios and Kaltschmitt 2013), from which wheat straw (WS) is generated with an annual output of over 2 million tons of dry matter (Valdez-Vazquez *et al*. 2010). With respect to its chemical composition, wheat straw is comprised of greater than 60% fermentable complex carbohydrates in the form of cellulose and

hemicellulose, which bind strongly to lignin, resulting in low biodegradability (Kaparaju *et al*. 2009). Several physical, chemical, and biological treatments and their combinations have been applied to increase the biodegradability and conversion of lignocellulosic biomass into methane, but most methods are expensive or unfavorable for the environment (Sawatdeenarunat *et al*. 2015).

Anaerobic digestion is performed using many microorganisms that coexist, forming a stable, self-regulating consortium (Valdez-Vazquez and Poggi-Varaldo 2009). To obtain complete substrate conversion and a high methane yield, specialized lignocellulolytic microorganisms require the optimal nutritional balance and conditions for methanogenic activity. Macronutrients, such as nitrogen (N) and phosphorous (P), must be kept at optimal ratios with respect to carbon. A high C:N ratio indicates a deficiency in N, such that microorganisms are unable to synthesize the enzymes that are required to hydrolyze and ferment the substrate. Conversely, a low C:N ratio allows the release of ammonium ions, increasing the pH and causing toxicity in the methanogenic population (Chandra *et al*. 2012). The optimal C:N ratio in anaerobic digesters is between 15 and 30, depending on the substrate (Mshandete *et al*. 2004; Wang *et al*. 2012). However, the N and P contents in LB vary widely. In wheat straw, N ranges between 3.5 and 11.6 g/kg, and P varies from 0.05 to 3.5 g/kg (De *et al*. 2003; Contreras-Ramos *et al*. 2004; Kaboneka *et al*. 2004; Bakisgan *et al*. 2009; Burhenne *et al*. 2013; Gupta *et al*. 2013; Krishania *et al*. 2013). These variations are attributed to crop age, climate, and nutrient availability in the soil (Bonde and Rosswall 1987; Takahashi and Anwar 2007). Because of these variations, it is necessary to study the effects of N and P levels on anaerobic digestion. Thus, co-digestion with other wastes is a useful strategy to balance these macronutrients. However, first it is necessary to study the N and P requirements for methane production from single wheat straw sources in its native form, as this knowledge can improve the design of anaerobic digesters using lignocellulosic substrates alone or co-digested with other materials. This study characterized a lignocellulolytic consortium using pyrosequencing to define the microbial diversity. This microbial population was used for the direct anaerobic digestion of untreated wheat straw. The C:N and C:P ratios were optimized for methane production using a response surface methodology based on a central composite design.

EXPERIMENTAL

Inoculum and Substrate

The inoculum consisted of anaerobic sludge from a 10-m^3 tubular plug flow digester fed with cow manure, the characteristics of which were previously described by Pérez-Rangel *et al*. (2015). Winter wheat (*Triticum aestivum* L.) was harvested mechanically using a cultivar (Urbina S2007) in Guanajuato, Mexico. Chopped wheat straw samples were processed as reported (Lara-Vázquez *et al*. 2014). Samples retained on a 3.35-mm sieve were tested. The wheat straw was washed with tap water and dried at 70 °C overnight. Components of the wheat straw on a per kilogram basis were determined according to methods previously reported (Allison 1965; Bartlett *et al.* 1994; Cunniff 1995; APHA 1999): 956 g of total solids, 867 g of volatile solids, 419 g of total organic carbon, 387 g of cellulose, 190 g of hemicellulose, 173 g of lignin, 4.4 g of total Kjeldahl nitrogen, 2.7 g of total P, and 86 g of ash.

DNA Extraction, Pyrosequencing, and Data Processing

Genomic DNA was extracted from 0.5 ± 1.0 mg of anaerobic sludge using the Power Soil DNA Extraction Kit® (Mo Bio Laboratories Inc., Carsbad, CA, USA), according to the manufacturer's instructions. Genomic DNA samples were sent to the Research and Testing Laboratory Company (RTL, Lubbock, TX, USA) for 16S rRNA gene amplification and pyrosequencing. The V1 to V3 region of the bacterial 16S rRNA gene was amplified with the primers 28F and 519R and subsequently sequenced at RTL (Dowd *et al*. 2008). Short and singleton sequences, noisy reads, and chimeric sequences were removed using the USEARCH algorithm and UCHIME software (drive5.com/index.htm) executed in *de novo* mode (Edgar 2010; Edgar *et al*. 2011). The minimum sequence length was 250 base pairs with a quality score of greater than 30. Operational taxonomic units (OTUs) were selected using the UPARSE algorithm (Edgar 2013). The USEARCH global alignment algorithm was used to assign the taxonomic identity for each of the OTUs against the RTL database of high quality sequences derived from the NCBI database (http://www.ncbi.nlm.nih.gov). Pyrosequencing results were displayed as KRONA charts by using the RTL server.

Optimization of C:N and C:P Ratios for Methane Production

Batch reactors were run in 120-mL serological bottles with a working volume of 100 mL. The reactors were loaded with 20 mL of inoculum and 5.0 ± 0.1 g of untreated wheat straw in an inoculum to substrate volatile solid ratio of 0.2 to 1.0. N- and P-free mineral medium was used to charge the reactors (composition in grams per liter: $NaHCO_3$, 10; $MgCl_2$, 0.1; $CaCl_2$, 0.020; $MnSO_4$, 0.015; $FeSO_4$, 0.025; $CuSO_4$, 0.005; $CaCl_2$, 0.000125). The initial pH was adjusted to 7.0, and the batch reactors were incubated statically at 37 °C for 30 days.

To maximize the biogas and methane production, the C:N and C:P ratios were studied as independent variables and adjusted per response surface methodology (RSM) using a central composite design. The C:N and C:P ratios were adjusted with urea and potassium phosphate monobasic, respectively. The N and P contents in the wheat straw were not considered in the adjustment since the availability of such nutrients was unknown, and it could be affected by factors not considered in this study (Soon and Arshad 2002). The biogas and methane yields, total volatile solid removal, final pH, and enzymatic activity were the dependent output responses. The experiments were performed in three replicates for the factorial and axial points and in five replicates for the central points to account for the nonadjustable data and to allow calculations *via* analysis of variance (ANOVA). The factors and levels of independent input variables are shown in Table 1.

Table 1. Levels of Factors Selected for the Central Composite Design

Factors	Levels				
	- 1.414	-1	0	1	1.414
C:N	2	31	101	171	200
C:P	5	77	253	428	500

To determine the endogenous methane production, two control treatments were used: control 1) with inoculum, N- and P-free mineral medium, and wheat straw, and control 2) with inoculum and N- and P-free mineral medium without straw. The relationship between the response output variables and the independent variables was expressed using a second-degree quadratic polynomial equation (Box *et al.* 1978). The

analysis of the response surface, ANOVA results, and optimal conditions were performed using Design Expert 8.0.7.1® (Stat-Ease Inc., Minneapolis, MN). The significant effects on dependent variables were determined using a *t*-test, with a probability value (*p*-value) of less than 0.05.

Analytical Methods

Biogas accumulation was measured periodically using the displacement of a lubricated syringe. Biogas composition (H_2, O_2, N_2, CO_2, and CH_4) was analyzed by gas chromatography on a Clarus 580 GC System (Perkin-Elmer, Waltham, MA), equipped with a thermal conductivity detector and a Molsieve column (30 m x 0.53 mm). The following program was run: oven 50 to 100 °C at a rate of 7 °C/min. The injector and detector temperatures were 150 and 200 °C, respectively. The total volatile solids (TVS) and pH were determined according to standard methods (APHA 1999). The cellulase activity (IU/mL) was determined in the reactor supernatants according to methods by Mandels and Weber (1969). For this enzyme assay, the controls included supernatant without substrate and substrate without supernatant.

RESULTS AND DISCUSSION

Taxonomic Composition of the Lignocellulolytic Consortium

Figure 1 shows the taxonomic composition and abundance of bacteria in the lignocellulolytic inoculum. A total of 9,987 sequences were obtained and compared with the database, and 36% of the sequences could not be taxonomically identified. The lack of knowledge of these species could be related to difficulties in their cultivation and study. Complementary studies are required to identify them and to establish their role in the microbial community. From the sequences with taxonomical affiliation, the anaerobic sludge comprised 14 phyla, of which *Firmicutes* and *Bacteroidetes* represented 67% of the inoculum.

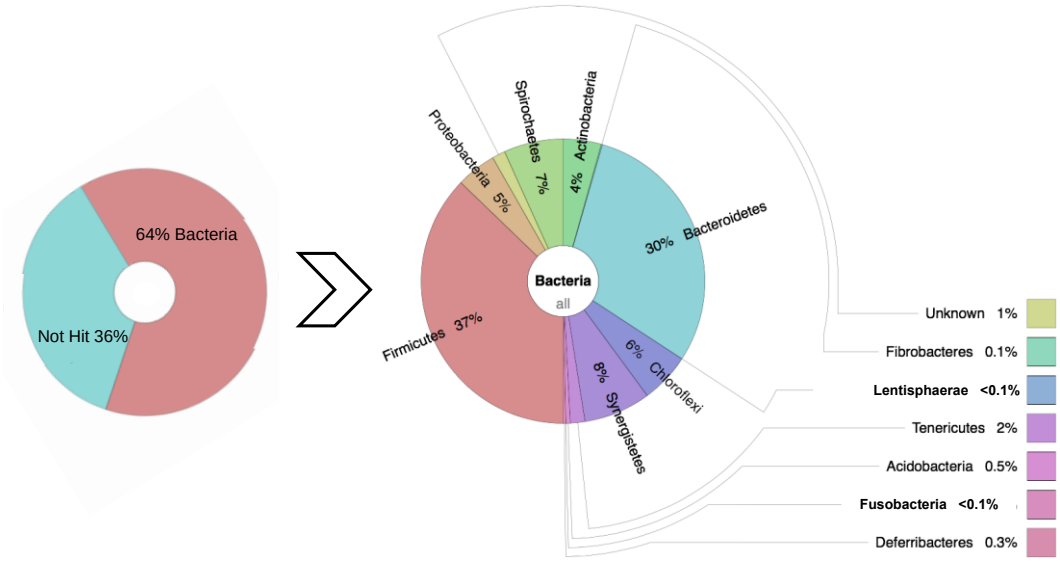

Fig. 1. Taxonomic classification and abundance of lignocellulolytic bacterial phyla

The distribution and abundance of the identified families revealed a well-balanced bacterial population (Fig. 2). Members of the families Ruminococcaceae (Latham and Wolin 1977), Clostridiaceae (Sleat *et al*. 1984), Bacteroidaceae (Murray *et al*. 1984), Bacillaceae (Robson and Chambliss 1984), and Fibrobacteraceae (Stewart and Flint 1989) are relevant hydrolytic species in anaerobic environments, with the potential for solubilizing lignocellulosic substrates.

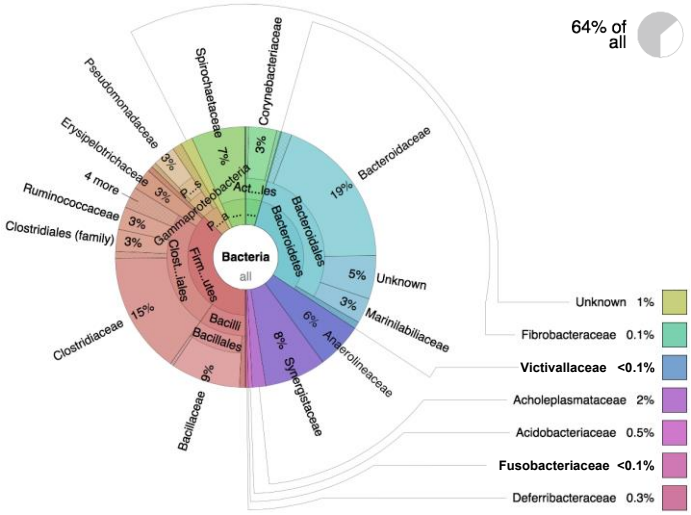

Fig. 2. Taxonomic classification and abundance of lignocellulolytic bacteria at the family level

Optimization of C:N and C:P Ratios for CH_4 Production from Wheat Straw

Kinetic behavior

Batch digesters produced biogas for 30 days to determine the methane potential from direct anaerobic digestion of a single untreated wheat straw sample. Representative treatments were characterized with regard to kinetic behaviors including biogas production, methane content, and the methane production in the digesters for the central points of the central composite design (C:N 101:1 and C:P 253:1). The results are shown with respect to conditions offering the best performance (C:N 31:1 and C:P 428:1), those that performed worst (C:N 2:1 and C:P 253:1), and the control 1 (with inoculum and wheat straw but without N or P adjustments) (Fig. 3). The control 2 (self-digestion of the inoculum) had a negligible biogas accumulation in the whole incubation time without methane content. For all of the treatments (except for C:N 2:1 and C:P 253:1), the biogas production by day 30 remained in the log phase, meaning that more time was required to attain complete digestion of the untreated wheat straw. However, the methane content had stabilized at 63% to 70% by day 14 in the digesters (C:N 31:1 and C:P 428:1) and by day 20 for the other treatments. Digesters with the highest N content (C:N 2:1 and C:P 253:1) showed erratic behavior between replicates, which was attributed to disrupted microbial activity. For example, some had high methane content before the other treatments (ranging from 23% to 63%). However, the high N level inhibited the biological process, resulting in low biogas production (320 mL/L) and, consequently, the lowest methane potential. The methane production was highly dependent on the N and P supplements. At C:N 31:1 and C:P 428:1, the lag phase declined from 10 to 6 days, and the methane potential rose from 0.091 to 0.197 m^3/kg TVS compared with the control treatment. When N and P were added in excess, methane production was nearly completely inhibited.

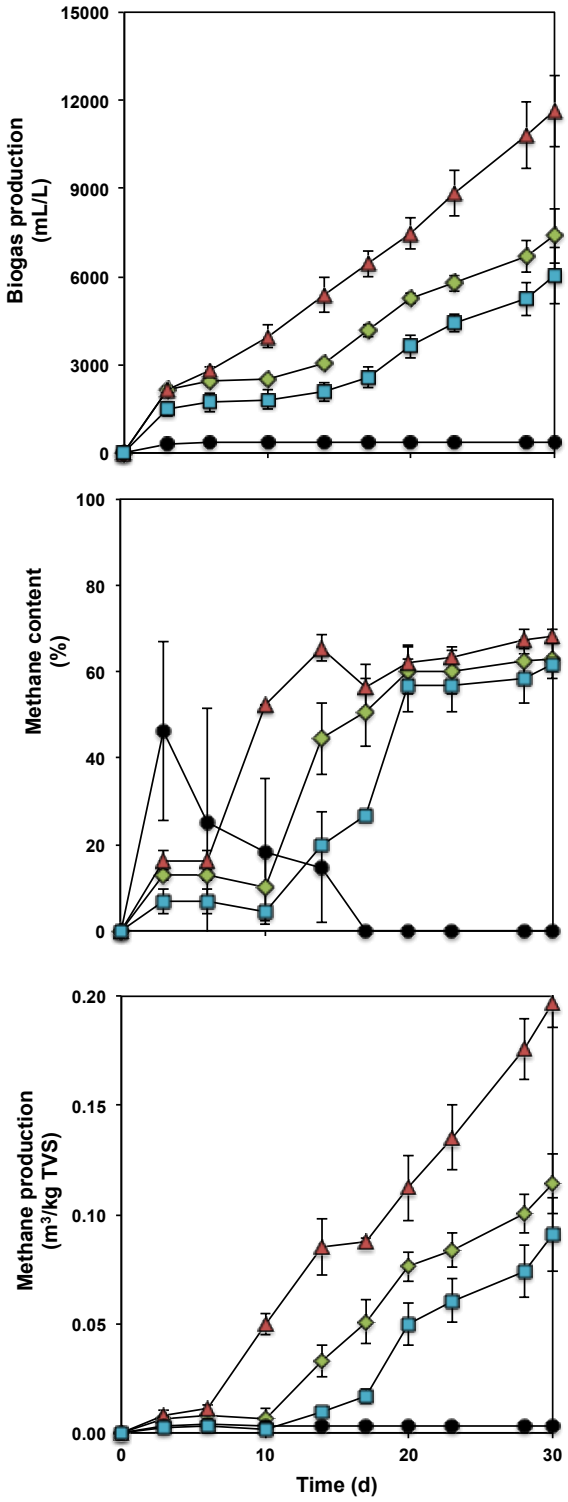

Fig. 3. Kinetic behavior of the direct anaerobic digestion of single untreated wheat straw with adjustment of the C:N and C:P ratios. Treatments: C:N 101:1 and C:P 253:1 (◆), C:N 31:1 and C:P 428:1 (▲), C:N 2:1 and C:P 253:1 (•), and control 1 (■). Results are average measurements based on three replicates for the treatment (n = 3) and five replicates for the central points (n = 5). The errors bars represent the standard deviation of the measurements**.**

Methane production

Table 2 shows the average values of methane production for each treatment. ANOVA showed that the C:N and C:P ratios had a highly significant effect on this dependent response ($p < 0.0001$). For methane production (m^3/kg TVS), regression analysis generated the following quadratic equation (in terms of coded factors):

$$\text{Ln } (CH_4 \text{ production}) = -2.18 + 0.96 X_1 + 0.75 X_2 - 1.13 X_1X_2 - 0.89 X_1^2 - 0.15 X_2^2 \quad (1)$$

The regression equation had an R^2 value of 0.86, consistent with the adjusted R^2 of 0.83 ($R^2 > 0.75$ indicates the aptness of the model), ensuring adequate adjustment of the experimental data to the quadratic model. The significant model terms were X_1, X_2, X_1X_2, and X_1^2 ($p < 0.0001$). The regression analysis of the model (Eq. 1) predicts a maximum methane potential of 0.27 m^3/kg TVS_{added} at C:N ratio of 76:1 and a C:P ratio of 500:1 after 30 days of incubation.

Figure 4a shows the response surface and contour plots of the methane production as a function of the C:N and C:P ratios. In addition to the peak of methane production, a longitudinal crest is visible. Here, higher methane production was attained beginning from the peak until C:N 200:1 and C:P 5:1, at which point the methane production was 0.18 m^3/kg TVS_{added}.

Total volatile solid removal

Table 2 shows the average TVS removal in the treatments and control. ANOVA showed that only the C:P ratio had a significant effect on this response variable ($p < 0.0001$). For TVS removal (%), the regression analysis generated the following equation (in terms of coded factors):

$$\text{TVS removal} = 21.72 + 0.21 X_1 + 6.58 X_2 - 4.01 X_1X_2 \quad (2)$$

The regression equation had an R^2 value of 0.84, which agreed reasonably with the adjusted R^2 of 0.81, ensuring adequate adjustment of the experimental data to the model. The significant model terms were X_2 and $X_1 X_2$ ($p < 0.0001$ and $p < 0.01$, respectively). Figure 4b shows the response surface and contour plot of TVS removal as a function of the C:N and C:P ratios. Notably, greater substrate consumption was reached when the C:P ratio was greater than 370:1 and C:N ratio greater than 101:1.

Cellulase activity

Table 2 shows the average cellulase activity in the supernatants of the treatments and control. By ANOVA, only C:N ratio had a significant effect on this response ($p < 0.0001$). For enzyme activity (IU/mL), the regression analysis generated the following equation (in terms of coded factors):

$$\text{Cellulase activity} = 0.41 - 0.16 X_1 + 0.06 X_2 - 0.01 X_1X_2 + 0.16 X_1^2 + 0.02 X_2^2 \quad (3)$$

The regression equation had an R^2 value of 0.86, which is in reasonable agreement with the adjusted R^2 of 0.81. The significant model terms were X_1 and X_1^2 ($p < 0.0001$). Figure 4c shows the response surface and contour plot of the cellulase activity as a function of the C:N and C:P ratios. Higher enzymatic activities were consistent with the C:N and C:P levels at which greater substrate consumption occurred (low C:N ratio and high C:P ratio).

Table 2. Experimental and Predicted Results of the Central Composite Design for Methane Production, Total Volatile Solid Removal, Cellulase Activity, and Final pH

No.	Coded levels		Actual values		Methane production (m^3/kg TVS)		TVS removal (%)		Cellulase activity (IU/mL)		Final pH	
	X_1	X_2	C:N	C:P	Exp.	Predicted	Exp.	Predicted	Exp.	Predicted	Exp.	Predicted
23, 28, 29	0	-1.414	101	5	0.044 ± 0.010	0.029	12 ± 0.4	12	0.31 ± 0.02	0.38	5.9 ± 0.13	5.8
2, 12, 14	-1.414	0	2	253	0.004 ± 0.001	0.005	20 ± 0.4	21	1.03 ± 0.02	0.96	8.8 ± 0.04	8.2
9, 17, 26	1	1	171	428	0.087 ± 0.012	0.071	29 ± 1.2	25	0.45 ± 0.01	0.48	6.2 ± 0.05	6.2
8, 18, 24	1	-1	171	77	0.094 ± 0.014	0.153	21 ± 1.4	19	0.47 ± 0.00	0.38	6.4 ± 0.05	6.2
5, 10, 21	0	1.414	101	500	0.139 ± 0.011	0.242	26 ± 1.0	31	0.64 ± 0.00	0.54	6.3 ± 0.55	6.0
1, 4, 13, 16, 20	0	0	101	253	0.114 ± 0.014	0.113	23 ± 0.8	22	0.41 ± 0.05	0.41	6.6 ± 0.14	6.6
11, 15, 22	1.414	0	200	253	0.097 ± 0.024	0.074	18 ± 1.6	22	0.47 ± 0.00	0.50	6.6 ± 0.22	6.8
3, 19, 27	-1	1	31	428	0.197 ± 0.011	0.100	35 ± 1.3	32	0.69 ± 0.06	0.81	6.8 ± 0.12	7.3
Control 1	-0.086	-0.557	95	155	0.091 ± 0.017	0.063	20 ± 2.3	18	0.18 ± 0.01	0.40	6.5 ± 0.19	6.5
Note: Results expressed as mean ± standard error												

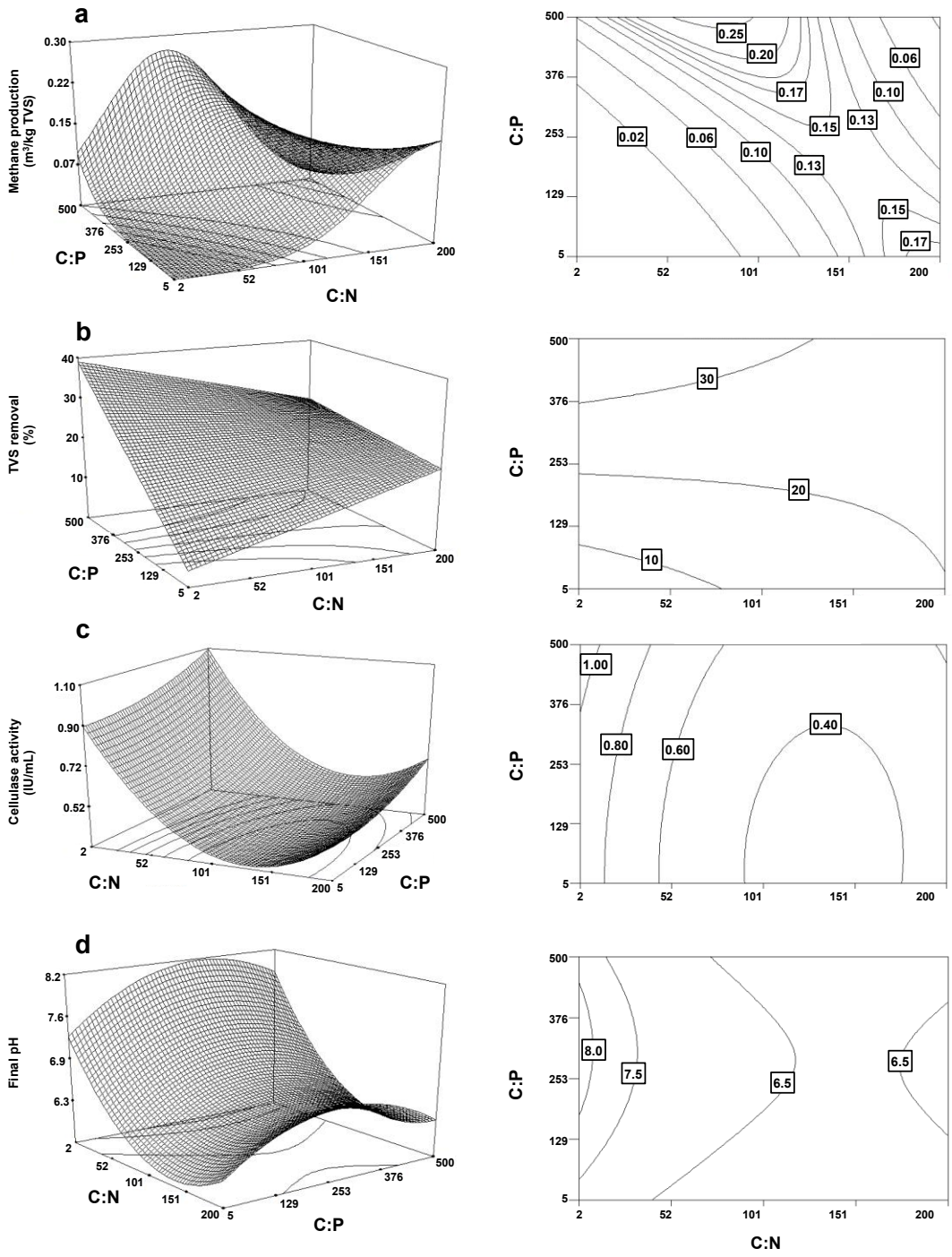

Fig. 4. Response surface and contour plots of (a) methane production (m^3/kg TVS), (b) total volatile solid removal (%), (c) cellulase activity (IU/mL) and (d) final pH as function of the C:N and C:P ratios from the anaerobic digestion of single wheat straw

Final pH

The final pH values after 30 days of anaerobic digestion of single, untreated wheat straw specimens are shown in Table 2. By ANOVA, only C:N ratio influenced this response ($p < 0.0001$). The regression analysis generated the following quadratic equation (in terms of coded factors):

$$\text{Final pH} = 6.59 - 0.5\,X_1 + 0.07\,X_2 - 0.06\,X_1X_2 + 0.45\,X_1^2 - 0.33\,X_2^2 \quad (4)$$

The R^2 value of 0.77, versus the adjusted R^2 of 0.72, ensured acceptable adjustment of the experimental data with the quadratic model. Two terms, X_1 and X_1X_2, were the most significant ($p < 0.001$). As shown in Fig. 4d, the pH exceeded 7.5 when the C:N ratio was greater than 30:1, wherein the treatment with C:N 2 and C:P 253 had a final pH of 8.8.

Of the various types of lignocellulosic substrate, wheat straw is one of the most recalcitrant to biological transformation (Sharma *et al.* 1988; Gunaseelan 1997; Liew *et al*. 2012). The methane production that typically occurs with untreated wheat straw ranges from 0.067 to 0.249 m^3/kg TVS, and greater production requires thermophilic incubation temperatures and conversion times of greater than 100 days (Table 3). Here, we examined a broad range of C:N and C:P ratios in the AD of untreated wheat straw to determine beneficial and inhibitory levels.

The optimal nutrient adjustment showed advantages in several ways. The microbial activity behaved better when nutrients were added than without nutrient balancing, thus the length of lag phase diminished by 40% (Fig. 3), and the methane production rate doubled to 0.006 m^3/kg TVS-d. As a result, the potential and productivity of methane production well exceeded the results of other studies using untreated wheat straw (Table 3). Indeed, methane production approximates the potential under a thermophilic range but was four times faster.

Table 3. Biochemical Methane Potential from Single, Untreated Wheat Straw

Methane Potential (m^3/kg TVS_{added})	Temperature (°C)	Incubation Time (d)	Ref.
0.304	55	112	Hashimoto 1986
0.249	37	56	Sharma *et al*. 1988
0.161	35	60	Møller *et al*. 2004
0.070	35	105	Krishania *et al*. 2013
0.067	37	30	Liew *et al*. 2012
0.270[a]	**35**	**30**	**This study**
0.197[b]	**35**	**30**	
0.091[c]	**35**	**30**	

Notes: [a] predicted methane production at C:N 76:1 and C:P 500:1;
[b] highest methane production observed at C:N 31:1 and C:P 428:1;
[c] control treatment with inoculum and wheat straw but without nutrient adjustment

Conversely, high N levels exhibited an inhibitory effect on the methanogenic activity, a phenomenon that is well known (Rajagopal *et al*. 2013). However, the hydrolytic bacterial activity and TVS removal reached its highest values in these conditions. Many hydrolytic enzymes from members of the families identified in our lignocellulolytic inoculum show high stability at basic pH. Examples include *Ruminococcus* (Ohmiya *et al*. 1982), *Clostridium* (Song *et al*. 1985), and *Bacteroides* (Huang *et al*. 1988). It seems that despite having no growth, the hydrolytic enzymes remained active in the treatment with high N and P levels (treatment at C:N 2:1 and C:P 253:1; Fig. 3), resulting in substrate degradation. When the ammonia levels became inhibitory to the methanogens, biogas production stopped, but hydrolytic activity and solid removal continued.

Activities of diverse microbial groups (hydrolytic and fermentative micro-organisms as well as methanogens) should be coordinated to degrade lignocellulosic biomass ending up in a CH_4-rich biogas. This study presents different response variables, revealing deeper information about the process. In this way, methane production, cellulase activity, and pH responded differently to the N levels. First, a C:N ratio of 2:1 promoted the highest hydrolytic activity, but the basic pH stopped the methanogenic activity. Then, at a C:N ratio of 31, the reactors produced the highest observed value of 0.197 $m^3/kg\ TVS_{added}$ in agreement with guidelines for anaerobic digesters (Deublein and Steinhauser 2008). In fact, the observed value doubled the predicted value. This fact could be related to the favorable conditions for expression and activity of hydrolytic enzymes besides an adequate pH of 6.8 for the growth of methanogens (Table 2). After, at a C:N ratio of 101, the observed value was 40% lower than the predicted value. Under such conditions, the hydrolytic activity diminished but above all an acidic pH of 6.3 could be the primary factor detrimental to the methanogens. By Eq. 1, the model predicted the highest methane production of 0.270 $m^3/kg\ TVS_{added}$ at a C:N ratio of 76. However, it seems that a cellulase activity ~ 0.8 IU/mL and a pH ~ 7.0 will be the prerequisites to achieve this methane potential.

Hydrolytic bacteria present in the inoculum such as *Ruminococcus*, *Clostridium*, and *Bacteroides* behaved better at high N levels (C:N ratio of 2 and 31) at both neutral or basic pH, which is justified by their requirements for enzyme synthesis. Contrary to this, methanogen activity was maximized at C:N ratios between 31 to 101 but at pH strictly between 6.8 and 7.3. As a result, predicted and observed values of methane production showed discrepancies if these conditions were not satisfied. A better knowledge of the requirements of microbial diversity and nutrients for the direct conversion of lignocellulosic feedstock into biomethane will lead to modern digesters with robust hydrolytic capacities. The solid retention times will be decreased and, therefore, the size and cost of such facilities. The results presented here demonstrate that an adequate inoculum and the optimal nutrient levels improved the performance of methane production from an untreated lignocellulosic feedstock.

CONCLUSIONS

1. The anaerobic sludge used as the inoculum comprised at least 46% of members belonging to the Ruminococcaceae, Clostridiaceae, Bacteroidaceae, Bacillaceae, and Fibrobacteraceae families, which participate in the solubilization of lignocellulosic biomass.

2. Nitrogen and phosphorus adjustments for the direct conversion of untreated wheat straw into biomethane accelerated the exponential phase and doubled the methane productivity compared with a control without macronutrient additions.
3. The maximum methane production of 0.197 m^3/kg TVS_{added} was observed at a C:N ratio of 76:1 and C:P ratio of 500:1 and was three times higher than the control treatment.
4. The highest C:N ratio of 2:1 stimulated hydrolytic activity and solid removal but led to a pH of greater than 8.0, which inhibited methanogenic activity.

ACKNOWLEDGMENTS

G. J. T.-A. is grateful for the CONACYT scholarship. The authors thank Dr. Arturo Sanchez, CINVESTAV, Guadalajara, for help with the Design Expert software and data analysis. The authors are grateful to Jaime Perez for the technical support. Financial support was received from CONACYT projects (Grant No. 168921 and 188432).

REFERENCES CITED

Allison, L. E. (1965). "Organic carbon," in: *Methods of Soil Analysis, Part 2*, C. A. Black (ed.), American Society of Agronomy, Madison, WI, pp. 1367-1378. DOI: 10.2134/agronmonogr9.2.2ed.c39

APHA. (1999). *Standard Methods for the Examination of Water and Wastewater*, 20th Edition, American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC.

Bakisgan, C., Gümrah-Dumanli, A., and Yürüm, Y. (2009). "Trace elements in Turkish biomass fuels: Ashes of wheat straw, olive bagasse and hazelnut shell," *Fuel* 88(10), 1842-1851. DOI: 10.1016/j.fuel.2009.04.027

Bartlett, G. N., Craze, B., Stone, M. J., and Crouch, R. (1994). *Guidelines for Analytical Laboratory Safety*, Department of Conservation and Land Management, Sydney, Australia.

Bonde, T. A., and Rosswall, T. (1987). "Seasonal variation of potentially mineralizable nitrogen in four cropping systems," *Soil Sci. Soc. Am. J.* 51(6), 1508-1514. DOI: 10.2136/sssaj1987.03615995005100060019x

Box, G. E. P., Hunter, W. G., and Hunter, J. S. (1978). *Statistics for Experimenters* (1st edition), Wiley & Sons, New York, NY.

Burhenne, L., Messmer, J., Aicher, T., and Laborie, M.-P. (2013). "The effect of the biomass components lignin, cellulose and hemicellulose on TGA and fixed bed pyrolysis," *J. Anal. Appl. Pyrol.* 101, 177-184. DOI: 10.1016/j.jaap.2013.01.012

Chandra, R., Takeuchi, H., and Hasegawa, T. (2012). "Methane production from lignocellulosic agricultural crop wastes: A review in context to second generation of biofuel production," *Renew. Sustain. Energy Rev.* 16(3), 1462-1476. DOI: 10.1016/j.rser.2011.11.035

Contreras-Ramos, S. M., Alvarez-Bernal, D., Trujillo-Tapia, N., and Dendooven, L. (2004). "Composting of tannery effluent with cow manure and wheat straw," *Bioresour. Technol.* 94(2), 223-228. DOI: 10.1016/j.biortech.2003.12.001

Cunniff, P. (1995). *Official Methods of Analysis of AOAC International* (16th Edition), Association of Official Agricultural Chemists, Washington, DC.

De, D., and Singh, G. P. (2003). "Effect of could process monensin enriched urea molasses mineral blocks on performance of crossbred calves fed a wheat straw based diet," *Anim. Feed Sci. Technol.* 103(1-4), 51-61. DOI: 10.1016/S0377-8401(02)00256-0

Deublein, D., and Steinhauser, A. (2008). *Biogas from Waste and Renewable Sources: An Introduction*, Wiley-VCH Verlag, Weinheim, Germany.

Dowd, S. E., Callaway, T. R., Wolcott, R. D., Sun, Y., McKeehan, T., Hagevoort, R. G., and Edrington, T. S. (2008). "Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP)," *BMC Microbio.* 8(1), 125. DOI: 10.1186/1471-2180-8-125

Edgar, R. C. (2010). "Search and clustering orders of magnitude faster than BLAST," *Bioinformatics* 26(19), 2460-2461. DOI: 10.1093/bioinformatics/btq461

Edgar, R. C. (2013). "UPARSE: Highly accurate OTU sequences from microbial amplicon reads," *Nat. Methods* 10(10), 996-998. DOI: 10.1038/nmeth.2604

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). "UCHIME improves sensitivity and speed of chimera detection," *Bioinformatics* 27(16), 2194-2200. DOI: 10.1093/bioinformatics/btr381

Gerardi, M. H. (2003). *The Microbiology of Anaerobic Digesters*, John Wiley & Sons, Hoboken, NJ. DOI: 10.1002/0471468967

Gunaseelan, V. N. (1997). "Anaerobic digestion of biomass for methane production: A review," *Biomass Bioenerg.* 13(1-2), 83-114. DOI: 10.1016/S0961-9534(97)00020-2

Gupta, A., Sharma, S., Saha, S., and Walia, S. (2013). "Yield and nutritional content of *Pleurotus sajor-caju* on wheat straw supplemented with raw and detoxified mahua cake," *Food Chem.* 141(4), 4231-4239. DOI: 10.1016/j.foodchem.2013.06.126

Hashimoto, A. G. (1986). "Pretreatment of wheat straw for fermentation to methane," *Biotechnol. Bioeng.* 28(12),1857-1866. DOI: 10.1002/bit.260281213

Huang, L., Forsberg, C. W., and Thomas, D. Y. (1988). "Purification and characterization of a chloride-stimulated cellobiosidase from *Bacteroides succinogenes* S85," *J. Bacteriol.* 170(7), 2923-2932, (http://jb.asm.org/content/170/7/2923.full.pdf).

Kaboneka, S., Nivyiza, J. C., and Sibomana, L. (2004). "Effects of nitrogen and phosphorus fertilizer addition on wheat straw carbon decomposition in a Burundi acidic soil," in: *Managing Nutrient Cycles to Sustain Soil Fertility in Sub-Saharan Africa*, A. Bationo (ed.), Academy Science Publishers, Kenya, pp. 151-162.

Kaparaju, P, Serrano, M., Thomsen, A. B., Kongjan, P., and Angelidaki, I. (2009). "Bioethanol, biohydrogen and biogas production from wheat straw in a biorefinery concept," *Bioresour. Technol.* 100(9), 2562-2568. DOI: 10.1016/j.biortech.2008.11.011

Krishania, M., Vijay, V. K., and Chandra, R. (2013). "Methane fermentation and kinetics of wheat straw pretreated substrates co-digested with cattle manure in batch assay," *Energy* 57, 359-367. DOI: 10.1016/j.energy.2013.05.028

Lara-Vázquez, A. R., Quiroz-Figueroa, F. R., Sánchez, A., and Valdez-Vazquez, I. (2014). "Particle size and hydration medium effects on hydration properties and sugar release of wheat straw fibers," *Biomass Bioenerg.* 68, 67-74. DOI: 10.1016/j.biombioe.2014.06.006

Latham, M. J., and Wolin, M. J. (1977). "Fermentation of cellulose by *Ruminococcus flavefaciens* in the presence and absence of *Methanobacterium ruminantium*," *Appl. Environ. Microbiol*. 34(3), 297-301, (http://aem.asm.org/content/34/3/297.long).

Liew, L. N., Shi, J., and Li, Y. (2012). "Methane production from solid-state anaerobic digestion of lignocellulosic biomass," *Biomass Bioenerg*. 46, 125-132. DOI: 10.1016/j.biombioe.2012.09.014

Mandels, M., and Weber, J. (1969). "The production of cellulases," in: *Cellulases and Their Applications*, Hajny, G. J., and Reese, E. T. (eds.), American Chemical Society, Washington, DC, pp. 391-414. DOI: 10.1021/ba-1969-0095.ch023

Møller, H. B., Sommer, S. G., and Ahring, B. K. (2004). "Methane productivity of manure, straw and solid fractions of manure," *Biomass Bioenerg.* 26(5), 485-495. DOI: 10.1016/j.biombioe.2003.08.008

Mshandete, A., Kivaisi, A., Rubindamayugi, M., and Mattiasson, B. (2004). "Anaerobic batch co-digestion of sisal pulp and fish wastes," *Bioresour. Technol.* 95(1), 19-24. DOI: 10.1016/j.biortech.2004.01.011

Murray, W. D., Sowden, L. C., and Colvin, J. R. (1984). "*Bacteroides cellulosolvens* sp. nov. A cellulolytic species from sewage sludge," *Int. J. Syst. Bacteriol.* 34(2),185-187. DOI: 10.1099/00207713-34-2-185

Ohmiya, K., Shimizu, M., Taya, M., and Shimizu, S. (1982). "Purification and properties of cellobiosidase from *Ruminococcus albus*," *J. Bacteriol.* 150(1), 407-409.

Pérez-Rangel, M., Quiroz-Figueroa, F. R., González-Castañeda, J., and Valdez-Vazquez, I. (2015). "Microscopic analysis of wheat straw cell wall degradation by microbial consortia for hydrogen production," *Int. J. Hydrogen Energy* 40(1),151-160. DOI: 10.1016/j.ijhydene.2014.10.050

Rajagopal, R., Masse, D. I., and Singh, G. (2013). "A critical review on inhibition of anaerobic digestion process by excess ammonia," *Bioresour. Technol.* 143, 632-641. DOI: 10.1016/j.biortech.2013.06.030

Rios, M., and Kaltschmitt, M. (2013). "Bioenergy potential in Mexico-Status and perspectives on a high spatial distribution," *Biomass Conv. Bioref*. 3(3), 239-254. DOI: 10.1007/s13399-013-0085-3

Robson, L. M., and Chambliss, G. H. (1984). "Characterization of the cellulolytic activity of a *Bacillus* isolate," *Appl. Environ. Microbiol*. 47(5), 1039-1046, http://aem.asm.org/content/47/5/1039.full.pdf

Sawatdeenarunat, C., Surendra, K. C., Takara, D., Oechsner, H., and Kumar Khanal, S. (2015). "Anaerobic digestion of lignocellulosic biomass: Challenges and opportunities," *Bioresour. Technol.* 178, 178-186. DOI: 10.1016/j.biortech.2014.09.103

Sharma, S. K., Mishra, I. M., Sharma, M. P., and Saini, J. S. (1988). "Effect of particle size on biogas generation from biomass residues," *Biomass* 17(4), 251-263. DOI: 10.1016/0144-4565(88)90107-2

Sleat, R., Mah, R.A. and Robinson, R. (1984). "Isolation and characterization of an anaerobic, cellulolytic bacterium, *Clostridium cellulovorans* sp. nov," *Appl. Environ. Microbiol.* 48(1), 88-93.

Song, F. L., Forsberg, C. W., and Gibbins, L. N. (1985). "Cellulolytic activity of *Clostridium acetobutylium*," *Appl. Environ. Microbiol.* 50(2), 220-228, (http://aem.asm.org/content/50/2/220.full.pdf).

Soon, K. Y., and Arshad, M. A. (2002). "Comparison of the decomposition and N and P mineralization of canola, pea and wheat residues," *Biol. Fertil. Soils* 36, 10-17. DOI: 10.1007/s00374-002-0518-9

Stewart, C. S., and Flint, H. J. (1989). "*Bacteroides (Fibrobacter) succinogenes*, a cellulolytic anaerobic bacterium from the gastrointestinal tract," *Appl. Microbiol. Biotechnol.* 30(5), 433-439. DOI: 10.1007/BF00263846

Takahashi, S., and Anwar, M. R. (2007). "Wheat grain yield, phosphorus uptake and soil phosphorus fraction after 23 years of annual fertilizer application to an Andosol," *Field Crops Res.* 101(2), 160-171. DOI: 10.1016/j.fcr.2006.11.003

Valdez-Vazquez, I., and Poggi-Varaldo, H. M. (2009). "Hydrogen production by fermentative consortia," *Renew. Sustain. Energy Rev.* 13(5), 1000-1013. DOI: 10.1016/j.rser.2008.03.003

Valdez-Vazquez, I., Acevedo-Benítez, J. A., and Hernández-Santiago, C. (2010). "Distribution and potential of bioenergy resources from agricultural activities in Mexico," *Renew. Sustain. Energy Rev.* 14(7), 2147-2153. DOI: 10.1016/j.rser.2010.03.034

Wang, X., Yang, G., Feng, Y., Ren, G., and Han, X. (2012). "Optimizing feeding composition and carbon-nitrogen ratios for improved methane yield during anaerobic co-digestion of dairy, chicken manure and wheat straw," *Bioresour. Technol.* 120, 78-83. DOI: 10.1016/j.biortech.2012.06.058

Article submitted: September 15, 2015; Peer review completed: December 19, 2015; Revised version received: January 13, 2016; Accepted: January 14, 2016; Published: March 24, 2016.
DOI: 10.15376/biores.11.2.4237-4251