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ORIGINAL ARTICLE

Hydrogen production by dark fermentation process from pig manure, cocoa mucilage, and coffee mucilage

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Abstract
The aim of this study was the evaluation of the influence of temperature, initial organic load, and C/N ratio in the biochemical hydrogen potential (BHP) by dark fermentation (DF) of mixtures composed of residual biomass derived from the Colombian agro-industry (pig manure, cocoa mucilage, and coffee mucilage) to contribute to residue management and the development of a bio-based economy in Colombia. A Box-Behnken experimental design was constructed, where the variables were as follows: three initial organic loads (2, 5, and 8 g COD/l), three C/N ratios (25, 35, and 45), and three different coffee and cocoa initial COD ratios (coffee mucilage (CFM):cocoa mucilage (CCM)) (1:3, 2:2, and 3:1). The experiments were carried out at thermophilic environments of 55 °C and 45 °C. Sludge from an anaerobic digester pretreated by thermal shock was used as an inoculum. The results of the experimental designs were used (one per temperature) to model the system mathematically. Also, a mathematical model MARS (multivariate adaptive regression splines) was used as an alternative method to corroborate the optimal points obtained. In both models, it was evidenced that at 55 °C and 45 °C, the best mixing conditions indicated a C/N ratio of 45 and the lowest CFM:CCM with a value close to 1:3. Likewise, it was evidenced that at the temperature of 55 °C, the optimum organic load is 8 g COD/l, while 45 °C is 2 g COD/l. Moreover, it was determined that CFM:CCM has less influence than the other independent variables studied, a factor that favors the country's scope, due to the availability of these two substrates is not permanent throughout the year.

Keywords Bio-hydrogen · coffee mucilage · cocoa mucilage · dark fermentation · mathematical model

1 Introduction

Fossil fuels are the energetic suppliers of more than 80% of the world demand [1]. Their use has caused global warming due to the emissions of CO_2, CH_4, and N_2O, in addition to the concern about the oil reserves depletion [2]. Due to this situation, many energetic alternatives had been investigated to guarantee the world energy supply and to develop sustainable systems that apply the circular economy concept using part of their residues to produce energy [3, 4].

Colombia is a country with high potential to produce energy transforming the biomass generated in the agricultural sector. In 2016, the Colombian agricultural industry generated more than 7.5 million tons of organic residues, with an increase of 0.5% since 2015 [5]. Cocoa and coffee are the primary crops in the country and the ones with higher export incomes [6]. Coffee and cocoa represent 9% and 7.6%, respectively, of the national agricultural gross national product (GNP), generating more than 800,000 direct and indirect jobs [7, 8]. However, there are not implemented alternatives for the treatment and valorization of the agricultural residues, and the producers are losing the profits and benefits from processes like anaerobic digestion and dark fermentation [9].

The annual Colombian coffee production in 2018 was 1,188,000 bags [10], and each bag weighs 125 kg. For each kilogram of coffee produced, it generates 0.5–0.6 l of coffee

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mucilage (CFM) [11] and, according to the Colombian national coffee federation, this residue is not treated or valorized in almost all the coffee farms [12]. There are few cases where this residue is treated by composting or anaerobic digestion but, considering the physicochemical properties, CFM is suitable to valorize it by dark fermentation.

Regarding the availability of cocoa mucilage (CCM), it is crucial to notice that the average annual cocoa production in Colombia was 46.000 ton in the past 10 years, with a constant increase thanks to new technologies to manage plagues, to reduce the costs of fertilizing and the introduction of genetic crops [13]. During the cocoa harvest and benefit, it is produced 0.39 kg of CCM per kilogram of the cocoa bean. Currently, this residue is not treated or valorized [14] and it is disposed in water bodies causing severe environmental problems. Similar to CFM, CCM has physicochemical properties such as a high percent of sugars [15].

The dark fermentation is a technology that produces hydrogen as a main energetic product. Hydrogen is energetically attractive due to its energy potential per unit of weight, corresponding to 120 MJ/kg, one of the highest of other fuels [16]. Additionally, its combustion produces mostly water, and in the production process, different products of interest for the chemical industry can be obtained [9]. Likewise, one of the most striking features of bio-hydrogen production lies in the consumption of residual biomass for its production through biological processes and the possibility of complementing other production systems [17].

In order to close the local production cycles, it is essential to determine the biochemical hydrogen potential (BHP) from residual biomass of agro-industrial systems in Colombia. Consequently, the present study focuses on the production of bio-hydrogen using as substrates CCM and CFM. Also, pig manure (PM) is used as a support in the biological process, being the primary nitrogen source, allowing to reach the adequate C/N ratio for the planned tests [18].

The presence of more than 4.1 million heads of pigs in the country [19] makes pig manure an alternative as a nitrogen source in the tests performed, considering that each lifting pig produces 2.35 kg of PM per day [20]. Although there are many farms in Colombia with anaerobic digesters and composting, not all the PM produced in the country can be valorized by these processes because crops near to pig farms do not demand a high amount of bio fertilizers [21, 22].

The use of these three substrates for H_2 production by dark fermentation has been studied by few researches. For coffee mucilage in combination with other substrates, productions are reported between 1.295 l H_2/l [23] and until 7.6 N l H_2/l_{CFM}d with PM [9], as well as a maximum production rate of 2.12 l $H_2/l_{solution}$ for pure CFM [18].

Hernández et al. (2014) studied DF processes using as substrate residues of the benefit of cocoa, finding a maximum production rate of 0.07 l $H_2/l_{solution}$ and 0.48 l $H_2/l_{solution}$; these results were obtained from a study before that [18]. PM has been studied under different conditions and combinations with other substrates. Therefore, the production rates have varied from study to study [24–26].

The main goal was to find the most workable mixture in terms of BHP through the development of a Box-Behnken design, where different operative variables were evaluated, and the optimization by the mathematical modeling of the system was constructed with the data found. Additionally, we provided information to contribute to overcoming the challenges that today face DF laboratory studies, including a perspective that will help with the industrial scale-up and for its final application in the generation of energy [14].

2 Methodology

2.1 Materials and methods

2.1.1 Substrates

The substrates selected were PM, collected at the Marengo Agricultural Research Center of the National University of Colombia, located in Mosquera (Cundinamarca). CCM—This residue was simulated in the laboratory from different references found of this industry and using cocoa bunches from private crops in Santander (Carmen de Chucurí) and Huila (Teruel). CFM came from private farms in the departments of Cundinamarca (Anolaima) and Huila (Teruel). All substrates kept at − 4 °C to avoid biochemical degradation by external agents. The substrates were diluted for the experimental design.

2.1.2 Inoculum

The inoculum came from an anaerobic digester of the company Alpina Productos Alimenticios S.A. located in Sopó (Cundinamarca). The digester works with waste water from the dairy industry and contains a wide range of anaerobic microorganisms and at neutral pH. The inoculum was stored at 4 °C before the experiments and brought to thermal shock, at boiling point for 30 min, to inhibit the growth of methanogenic microorganisms [27].

All experimental tests were performed using a substrate/inoculum ratio (S/X) equal to 1. Substrate was considered in grams of COD loaded in the reactor, and inoculum was quantified in grams of VS. Once the work units were established, the calculation was performed using the inoculum characterization to determine the inoculum volume needed for each combination.

2.1.3 Experimental design

A Box-Behnken experimental design was constructed to evaluate the effect of independent variables on H_2 production, the design was evaluated for two thermophilic conditions of 55 °C and the second for 45 °C. Three independent variables were established: coffee and cocoa mucilage ratio (CFM:CCM), substrate concentration, and C/N ratio, each with three own levels, as shown in Table 1. The parallel use of a 45 °C environment is due to studies that have reported hydrogen production at this temperature [28].

Colombia produces coffee and cocoa, but both are seasonal and the availability of the CFM and CCM changes during the year, that is the main reason to include the CFM:CCM.

Once the CFM:CCM ratio was defined, the amount of PM was calculated in order to adjust the C/N ratio of the experimental design; this is because PM is the source of nitrogen.

The initial concentration and the C/N ratio were adjusted according to the physicochemical characteristics of each substrate and its proportion in the mixture. The Box-Behnken experimental design evaluates 13 mixtures as is seen in Table 1. Each combination was performed by triplicate for a total of 39 reactors plus three targets, working through a discontinuous regime system. The required amount of each substrate and inoculum was placed in 250-ml amber bottles, the working volume (200 ml) was completed with distilled water, and 10 ml of 5.5 buffer solution. HCl 37% were used to adjust the pH to the reactors, which started with a pH of 5.5 ± 0.2.

The reactors were placed in a thermostated bath to maintain a thermophilic environment (55 °C and 45 °C ± 0.2) during the experimental time. The production of hydrogen was measured through the volume displacement of amber bottles with a capacity of 1 l, with a 0.5-M NaOH solution, which works as a CO_2 trap [29].

The test was monitored continuously to verify that there was no production of CH_4 in the biogas; this was done by daily monitoring with a Biogas 5000, a portable gas analyzer. The test was executed until the hydrogen production rate decreased. Finally, the information collected within the design of experiments was analyzed to determine the mixtures with the highest biohydrogen production potential, using the Box-Behnken model and the mathematical model of the MARS (multivariate adaptive regression splines).

2.1.4 Statistical analysis

From the experimental design, second order polynomial models were built that involve independent variables [30]. The model used is presented below:

$$Z = a_0 + \sum_{i=1}^{n} b_i x_{ni} + \sum_{i=1;j=1}^{n} d_{ij} X_{ni} X_{nj} \qquad (i < j)$$

where Z and X_{ni} are dependent and normalized independent variables, respectively; a_0 is a constant; and b_i, c_i, d_{ij} are the regression coefficients obtained from experimental data. Independent variables were normalized (X_n) by using the following equation:

$$X_n = \frac{(X - X_{\text{mean}})}{\left(\frac{(X_{\max} - X_{\min})}{2}\right)}$$

where X is the absolute value of the independent variable concerned, X_{mean} is the average value of the variable, and $X_{\max}$ and $X_{\min}$ are their maximum and minimum values, respectively.

2.1.5 Regression analysis

MARS is a nonparametric regression model, used for the analysis of time series proposed by Friedman. This model makes predictions about nonlinear relationships of the independent variables of the system, to obtain the modeling of the dependent variable with low error, using base sets of activation functions and their coefficients [31]. The MARS model has the following form:

$$\hat{f}(x) = a_o + \sum_{\text{Km}=1} f_i(X_i) + \sum_{\text{Km}=2} f_{ij}(X_i, X_j) + \sum_{\text{Km}=3} f_{ijk}(X_i, X_j, X_k) + \cdots$$

The first stage contemplates the sum of all functions related to a single variable; the next sum involves all the essential functions that involve two variables and successively [32].

Table 1 Experimental design

Combination	CFM:CCM*	(g COD/l)	C/N
1	3:1	2	35
2	1:3	2	35
3	3:1	8	35
4	1:3	8	35
5	3:1	5	25
6	1:3	5	25
7	3:1	5	45
8	1:3	5	45
9	2:2	2	25
10	2:2	8	25
11	2:2	2	45
12	2:2	8	45
13	2:2	5	35

* Units are expressed as g COD of CFM/g COD of CCM

2.1.6 Analytical methods

The characterization of the substrates and the digested mixtures of the experimental design were carried out as follows: the pH measurements were completed using an Edge Model HI2002 potentiometer. The total solids (TS) were completed by following the method 2540B APHA SM, in which the samples were taken to 105 °C for 24 h. The volatile solids (VS) were determined on a wet basis applying ASTM D3174, where the sample was ignited at 550 °C for 1 h. Kjeldhal total nitrogen (KTN) was performed following ASTM D1426, performing three intervals of 125 °C (20 min), 300 °C (30 min), and 400 °C (60 min); H_2SO_4 98% was used for digestion, and subsequently, it was distilled with concentrated NaOH and titrated with 0.1 N hydrochloric acid. The VFA and alkalinity analyses were developed according to the 5560D APHA SM and 2320B APHA SM respectively and the analyses for CODs using ASTM D1252-06 employing a Hanna kit and reactor.

The results of the characterizations of the substrates are presented in Table 2; these were obtained following the protocols established, except for the determination of carbohydrates, which was determined according to the method suggested by Albalasmeh [33].

3 Results and discussion

3.1 Substrates characterization and inoculum

Table 2 presents the results of the physical-chemical characterization. In order to validate the data obtained, these were compared with those of the database "database for biomass and waste" [34], finding that they are quite similar. The three substrates have high values of VS/TS as an indicator of the high organic matter content, their biodegradability, and valorization potential. Regarding carbohydrates, CCM and CFM reported high values and low content of proteins as Vriesmann [35]. On the other hand, the PM has a good percentage of proteins and to be the nitrogen source in the DF process [9].

3.2 Box-Behnken combinations

The time in all the experiments of 55 °C was 120 h, which is relatively short considering the high amount of soluble matter in the mixes evaluated but in concordance with the times reported in the literature [25, 36, 37]. In the experiments of 45 °C, the time was of 192 h; the difference in time of both temperatures is related to enzymatic hydrolysis, which is affected by the incidence of temperature [38]. Figure 1 shows the cumulative hydrogen production for each combination evaluated and for each temperature.

Temperature of 55 °C presented that the combination 4 has the highest yield with 90 ml H_2/day, which is equivalent to 284.02 ml H_2/g COD_{added}, showing a direct relationship between the production and the substrate concentrations [36]; combination 4 has an organic load of 8 g COD/l, a relation CFM:CCM of 1, and a C/N ratio of 35. However, combinations 8 and 12 reported significant hydrogen productions with lower substrate concentration by combination 8 (5 g COD/l) and a higher C/N ratio (45). The results obtained shows that higher hydrogen productions are achieved with 8 g COD/l for the substrates evaluated and when the C/N ratio is 45. High substrate concentrations allow for processing and valorizing higher amounts of residual biomass, avoiding the negative environmental impacts that these residues generate when they are not disposed of thoroughly.

On the other hand, the results for the experiments carried out at 45 °C described that the combination 11 has the highest yield with 38.5 ml H_2/day, equivalent to 244.5 ml H_2/g COD_{added}, less than reported at 55 °C. Combination 11 has an organic load of 2 g COD/l, a relation CFM:CCM of 1, and a C/N ratio of 45. However, combinations 8 and 12 reported significant hydrogen productions with higher substrate concentration (5 and 8 g COD/l) and the same C/N ratio (45) in both cases.

The Pareto analysis was performed to quantify the influence of these three variables on hydrogen production. Figure 2a shows that the importance of C/N and CFM:CCM is less than 20%, and the organic loading concentration is the variable with higher impact in the hydrogen production (83%); this agrees with that described by other authors, where higher concentrations increase the hydrogen production [18, 39, 40]. Figure 2b shows that the influence of CFM:CCM and concentration is minimal; in contrast, the C/N ratio is the variable with a more significant impact in the hydrogen yield (near 90%). The C/N ratio is an essential variable in DF processes because it is the measure of the presence of nitrogen in the reactor; the high or low presence of nitrogen can inhibit the microorganism [41].

From the results obtained in both experimental designs developed, it can be concluded that the range of substrate concentrations evaluated does not cause any toxic effect during the biological process. Results from both temperatures show a minimal influence of the CFM:CCM. The Pareto result about CFM:CCM was found relevant, considering that the coffee and cocoa are seasonal crops in Colombia, so the availability of these two residues change across the year.

3.3 Effluent characterization

The pH has the most significant influence on the effluent composition of the acidogenic reactors, and an increment implies lower hydrogen production [42, 43]. The initial pH was settled at 5.5, and at the end, the values measured were

Table 2 Characterization of the residual biomass used in the study

	Substrates			
	PM	CCM	CFM	Inoculum
TS	29.08 ± 0.45	19.7 ± 0.52	2.1 ± 0.14	5.6 ± 0.25
VS	22.92 ± 0.96	19.19 ± 0.53	1.82 ± 0.15	4.94 ± 0.18
NTK	2.10 ± 0.5	0.58 ± 0.6	0.31 ± 1.2	
Organic matter	71 ± 4.5	79.6 ± 3	97 ± 5.7	96.6 ± 5.2
COD	23.87 ± 9.65	10.50 ± 5.6	21.75 ± 12.5	
Proteins	22 ± 0.432	4 ± 0.737	6.5 ± 0.899	
Carbohydrates	2.9 ± 0.12	60.37 ± 0.25	85.95 ± 0.55	

All values are expressed in percent except for the COD (g/l); the analyses were performed on a wet basis

between 4.8 and 5.9. All the reactors of 55 °C presented pH values near to 5.5, with a tendency to decrease over time, which indicates that the experimental design continued to produce hydrogen and VFA. This behavior is in concordance with the data reported in the literature [44, 45]. At 45 °C, the trend was towards alkaline environment with pH between 5.5 and 6.5; this explains the decrease in hydrogen production that is evident against the thermophilic environment of 55 °C.

Figure 3 presents the relationship between VFA production and hydrogen production. The results obtained are in concordance with Agler [46], who reported that VFA production is directly related to hydrogen consumption for the generation of by-products in the fermentation processes. This situation is also reflected in what was reported by Nuo Liu et al. in 2018, where it justifies a lower yield in H_2 production due to the incidence of accumulation of VFA in the medium as an inhibitor of H_2 producers [47].

Likewise, it has been corroborated that the production of VFA interferes with the production of H_2 due to factors such as the dissociation of molecules and the consumption of hydrogen by homoacetogenic microorganisms [48]. This is evident in combinations such as 4 and 9 with higher hydrogen production and low VFA production. Contrary, combinations 2, 3, and 6 reported VFA productions higher than the media.

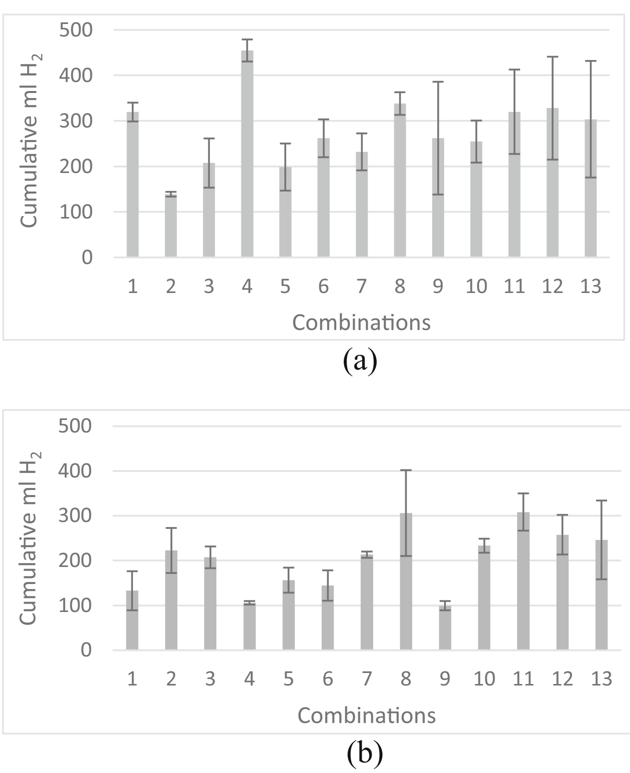

Fig. 1 Cumulative production of each of the combinations given in milliliters of H_2. **a** 55 °C. **b** 45 °C

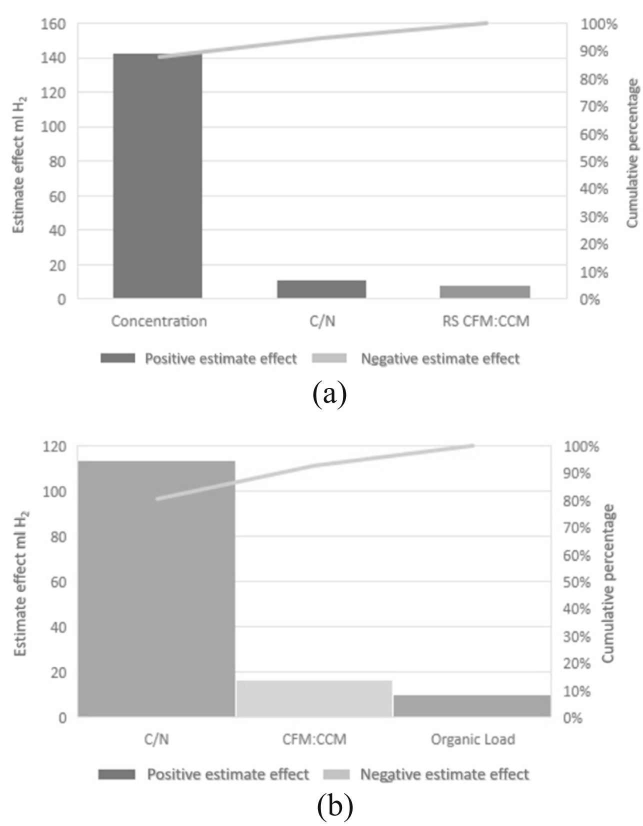

Fig. 2 Effects of the independent variables on the BHP. **a** 55 °C. **b** 45 °C

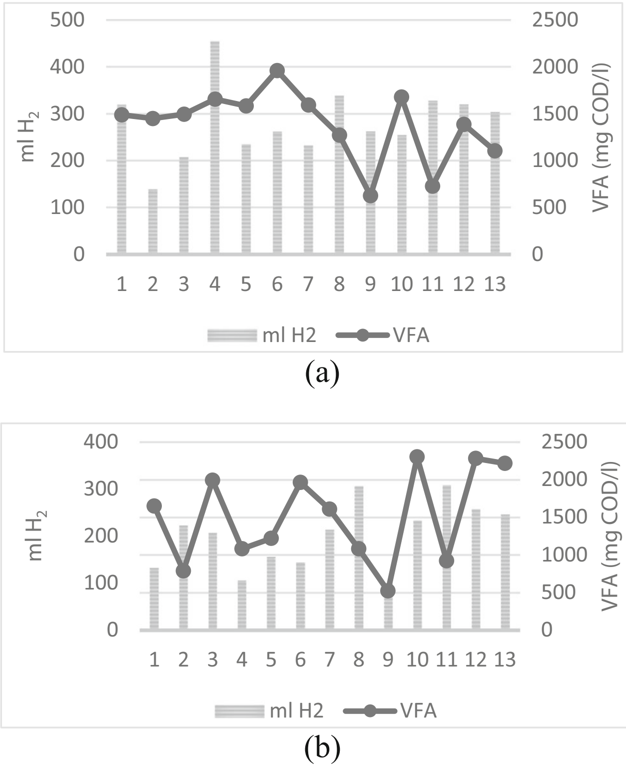

Fig. 3 Comparison between the production of hydrogen and VFA for each of the 13 combinations. **a** 55 °C. **b** 45 °C

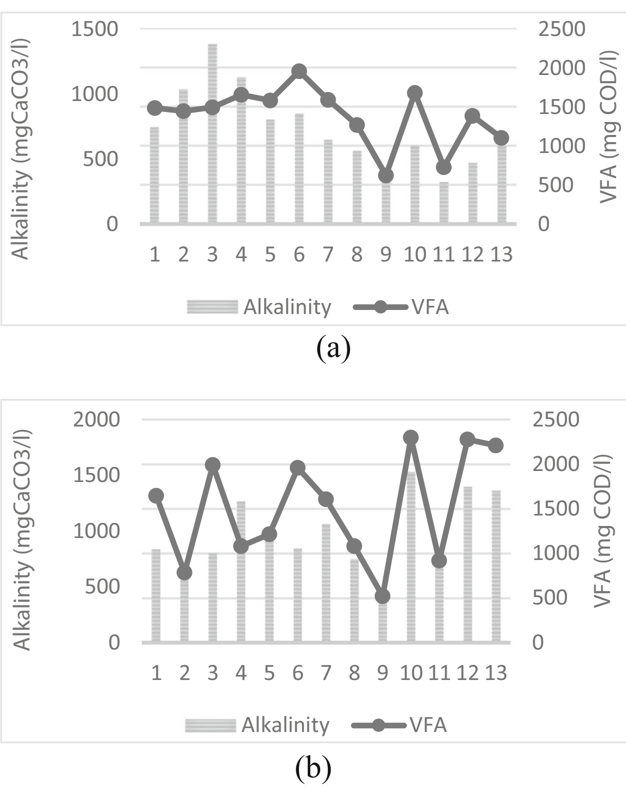

Fig. 4 Relationship between alkalinity and VFA production for each of the 13 mixtures. **a** 55 °C. **b** 45 °C

At the temperature of 45 °C in general, VFA production was relatively high and hydrogen production was low with an average production over 1500 mg COD/l.

The alkalinity is a desired effect between the reactors since it is an indicator of the buffer effect that the mixture possesses. Controlling the pH is fundamental since it has the most significant influence on the behavior of DF reactors; likewise, its direct influence on hydrogenase enzymes has been observed [49]. Also, high alkalinity values are desired, because controlling the pH in real time for industrial applications, adding acidic or basic solutions, is a challenge [50].

Figure 4 shows the relationship between alkalinity and VFA production, which indicates that in mixtures 3 and 4 (at 55 °C), there is a higher alkalinity of the mixture compared to the production of VFA, with a close VFA/alk ratio to 1:1; this is desirable because a high alkalinity favors the production of hydrogen due to the low production of VFA [51]. Regarding combination 6, it is shown that it has lower values of alkalinity that

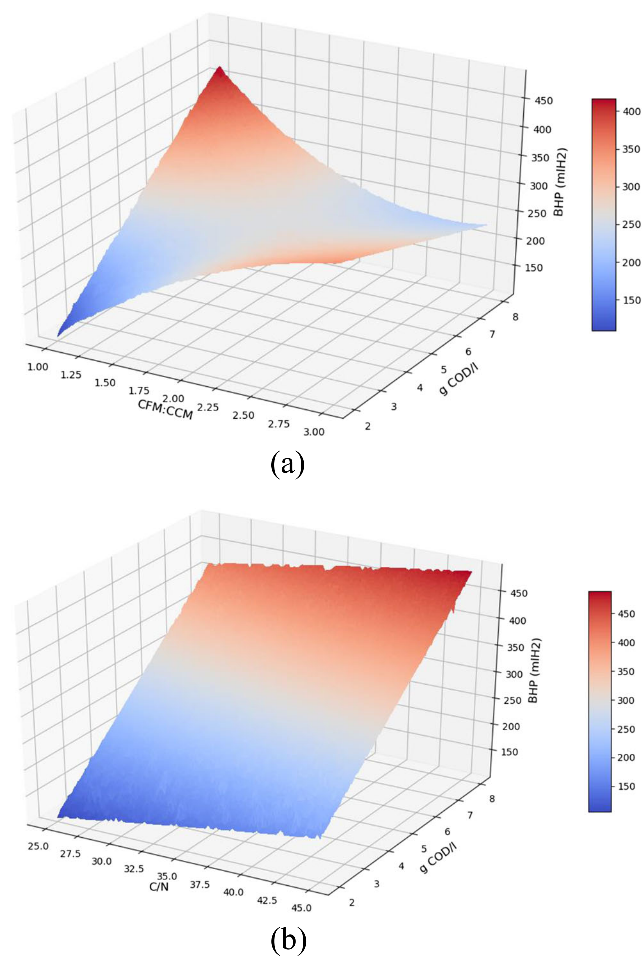

Fig. 5 Surface diagram for the BHP, based on the relation of the variables. **a** Substrate concentration vs. CFM:CCM ratio. **b** C/N vs. substrate concentration

can endanger the behavior of the process, considering that the VFA are the higher.

At the end of the tests, the % COD of removal of each mixture was determined. For 55 °C, it was found that the highest removal percentage corresponded to the load of 2 g COD/l with a maximum of 90%. Likewise, the lowest rate of organic matter turnover was found in loads of 8 g COD/l, corresponding to 20%. These percentages coincide with those reported by other authors, where low organic loads are related to high removal rates as the inoculum-substrate interaction is favored [51].

Likewise, other authors, such as Ghimire, state that a higher load has higher hydrogen production, but there is a lower percentage of COD removal [52]. Tests at 45 °C have an average COD removal of 55%.

The results at 55 °C have a lower average VFA production and higher hydrogen production yields. It is estimated that the 55 °C process takes place in the stage known as primary fermentation, while in the 45 test, the secondary fermentation is achieved. This can be evidenced in the high VFA production and low hydrogen yields [46, 53], compared with the 55 °C environment data.

3.4 Statistical analysis

3.4.1 Box-Behnken

The data obtained from the experimental assessment of the Box-Behnken design was assessed through the software STAGRAPHICS which generates a prediction of the BHP behavior. This is as a result of second-order polynomials, which enable the maximization of the BHP, by taking as variables those presented in the design, all of them related to each other, see Eqs. 1 and 2, where the substrate concentration is represented by [ ] symbol.

$$\begin{aligned}55°\mathrm{C\ ml\ H_2} = &-523.34 + 318.378 \times \mathrm{CFM:CCM} + 78.7218\\ &\times [\,] + 15.5558 \times \mathrm{C/N} - 23.9063 \times \mathrm{CFM:CCM}^2\\ &-35.6408 \times \mathrm{CFM:CCM} \times [\,] - 1.98475\\ &\times \mathrm{CFM:CCM} \times \mathrm{C/N} + 0.0581944 \times [\,]^2\\ &-0.00558333 \times [\,] \times \mathrm{C/N} - 0.128563 \times \mathrm{C/N}^2 \quad r^2: 0.90\end{aligned} \tag{1}$$

$$\begin{aligned}45°\mathrm{C\ ml\ H_2} = &-414.653 + 198.5 \times \mathrm{CFM:CCM} + 56.1944\\ &\times [\,] + 12.9417 \times \mathrm{C/N} - 49.0\\ &\times \mathrm{RS\ CFM:CCM}^2 + 16.0 \times \mathrm{CFM:CCM}\\ &\times [\,] - 2.6 \times \mathrm{CFM:CCM} \times \mathrm{C/N} - 3.27778\\ &\times [\,]^2 - 1.53333 \times [\,] \times \mathrm{C/N} + 0.08 \times \mathrm{C/N}^2 \quad r^2: 0.95\end{aligned} \tag{2}$$

The equation obtained for the 55 °C experiments presents a correlation coefficient capable of explaining 90.2% of the variability associated with BHP; also, the Durbin-Watson statistic ($D = 2.39$) shows that there is not a sub-estimation in the correlation coefficient. The model allowed the definition of an optimal combination, which has a concentration of 8 g COD/l, CFM:CCM of 1.02, and C/N ratio of 45, predicting a maximum production of 465.5 ml H_2.

Likewise, 45 °C presents a correlation capable of 95% and a similar Durbin-Watson statistic (2.83). The optimal combination, which has a concentration of 2 g COD/l, CFM:CCM of 1156, and C/N ratio of 45, predicts a maximum production of 356.7 ml H_2, which is close to the mixture with the highest production in the design.

The model equations obtained by the MARS regression, the next equation, have a correlation coefficient of 87% for the variability associated with the BHP in 55 °C (Eq. 3) and 73% in 45 °C (Eq. 4), similar to that obtained through the mathematical model of the Box-Behnken.

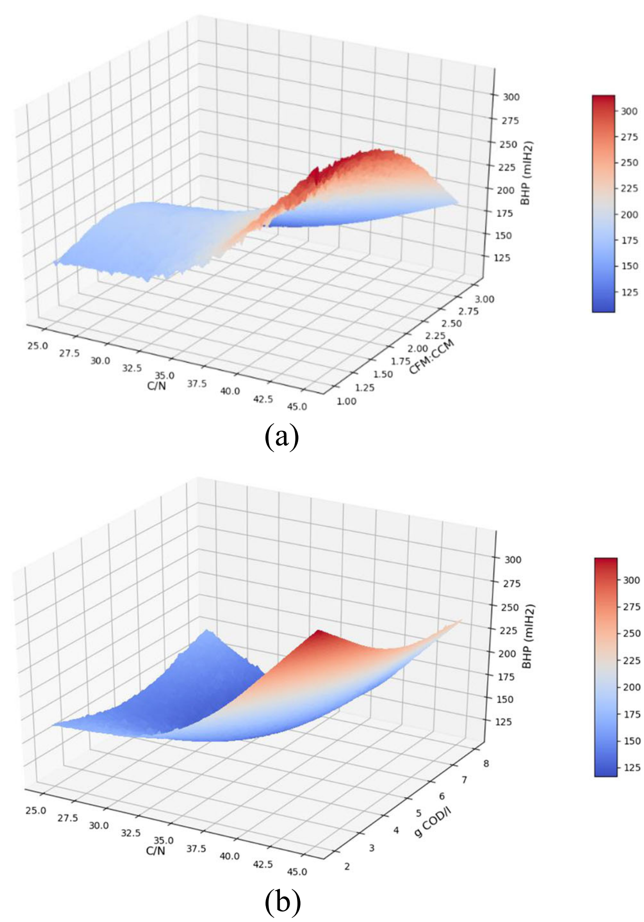

Fig. 6 Surface diagram for the BHP, based on the relation of the variables. **a** Substrate concentration vs. C/N ratio. **b** CFM:CCM vs. C/N ratio in 45 °C

$$55°\text{C ml } H_2 = -365.339 - 1.89522x_2 + 140.248x_1 - 105.938x_0x_1 + 246.789x_0 + 10.1636x_0x_2 - 2.634x_0x_0x_2 + 176,312x_0x_0x_1 - 0.028364x_2x_0x_2. \quad (3)$$

$$45°\text{C ml } H_2 = 0.00386733 + 0.0629226x_2 + 0.691673x_2x_2 - 0.0460999x_2x_2x_2 - 0.0460999x_2x_2x_2 + 0.000247229x_1x_2x_2x_2 - 3.85183^{-5}x_1x_1x_2x_2x_2 - 1.9362^{-6}x_0x_1x_1x_2x_2x_2 + 1.21749^{-7}x_1x_0x_1x_1x_2x_2x_2 - 54.8654x_1 + 4.89428x_1x_1 + 3.15187^{-5}x_0x_0x_1x_1x_2x_2x_2 + 3.15187^{-5}x_0x_0x_1x_1x_2x_2x_2 + 0.00024723x_1x_2x_2x_2 - 2.06359^{-5}x_0x_0x_0x_1x_1x_2x_2x_2 - 2.25641^{-6}x_2x_0x_1x_1x_2x_2x_2 + 0.691673x_2x_2 + 0.691673x_2x_2 - 1.93633^{-6}x_0x_1x_1x_2x_2x_2 - 0.851293x_0x_2 + 0.00110987x_2x_2x_2x_2 + 1.21753^{-7}x_1x_0x_1x_1x_2x_2x_2 - 3.85241^{-5}x_1x_1x_2x_2x_2 + 0.00024723x_1x_2x_2x_2 + 3.15187^{-5}x_0x_0x_1x_1x_2x_2x_2 - 1.93633^{-6}x_0x_1x_1x_2x_2x_2 \quad (4)$$

x_0: CFM:CCM, x_1: substrate concentration, and x_2: C/N ratio.

Using the MARS regression, it was obtained that the maximum possible cumulative production is equivalent to 489,592 ml H_2 with an initial concentration of 8 g COD/l, C/N close to 45, and an CFM:CCM of 1; this is consistent with pareto, since there is no evident influence of this relationship on the potential. In 45 °C, the better combination was concentration of 2 g COD/l, C/N 45, and CFM:CCM of 1, very similar with 55 °C. The main difference is concentration influenced by HRT of the experiment.

Figure 5 shows surface diagrams of different relations of the independent variables and their influence on the BHP-dependent variable. In Fig. 5a, it is observed that the relation between the highest concentration and the CFM:CCM of 1:3 favors the production of hydrogen. Figure 5b shows that a high organic load favors the production of hydrogen, without being greatly affected by the C/N.

Figure 6 shows surface diagrams of different relations of the independent variables and their influence on the BHP-dependent variable. In Fig. 6a, it is observed that the relation between the highest C/N and the lowest CFM:CCM of 1:3 favor the production of hydrogen. Figure 6b b shows that the best organic load has a value of 2 and C/N of 45, BHP decrease in relation with C/N ratio. The values for C/N and RS CFM:CCM that were presented concerning the concentration are supported and correspond to what is presented in the temperature of 55 °C.

4 Conclusions

According to the mathematical models of Box-Behnken and MARS at the optimal points, the BHP can be raised around 93 ml H_2/day and 44.6 ml H_2/day, respectively. Both models evidenced that at 55 °C and 45 °C, the best mixing conditions indicated a C/N ratio of 45 and the lowest CFM:CCM with a value close to 1. Likewise, the optimum organic load changes with the temperature; for 55 °C, the best concentration is 8 g COD/l, while for 45 °C is 2 g COD/l. On behalf of CFM:CCM, mixtures with a significant content of CCM produce more quantity of hydrogen due to the higher content of carbohydrates of this substrate.

Due to the results obtained regarding BHP, VFA concentration, and alkalinity for both experimental designs, it was concluded that the best working condition, in this case, is the temperature of 55 °C. Besides, the COD removals allow suggesting secondary processes associated with biorefinery schemes, which allows higher removals of COD and the further generation of value-added sub-products. All the substrates used are susceptible to be valorized by waste to energy technologies, decreasing the associated carbon footprint. An economic and environmental evaluation is necessary to determine the real potential for recovery.

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