Anaerobic Thermophilic Co-digestion of Sugarcane Biorefinery Residues Enhances Biogas Production

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ABSTRACT

This work evaluated the methane (CH_4) production potential from residues of integrated 1st (vinasse and filter cake) and 2nd (deacetylation pretreatment liquor from straw) generation (1G2G) sugarcane biorefinery. The small-scale study provided fundamentals for basing the optimization of co-digestion by assessing the best co-substrates synergistic conditions. Biochemical Methane Potential (BMP) tests showed co-digestion enhanced CH_4 yield of isolated substrates, reaching 605 $NmLCH_4$ gVS^{-1}. Vinasse and deacetylation liquor as the only co-substrates increased the BMP by 37.72%, indicating that the association of these two residues provided positive effects for co-digestion by nutritionaly benefeting the methanogenic activity. The filter cake had the lowest BMP (260 $NmLCH_4$ gVS^{-1}) and digestibility ($\leq$40%), being the stirring required to improve the mass transfer of biochemical reactions. The alkaline characteristic of the liquor (pH-12) prevented alkalinizers from being added to the co-digestion, which could be a relevant economic advantage for the implementation of the process in an industrial scale. The co-digestion system has proven to efficiently maximize waste management in the 1G2G sugarcane biorefineries and potentially enhance their energy generation (by at least in 18%), providing experimental elements for placing the biogas production as the hub of the bioeconomy in the agroindustrial sector.

Keywords: biogas; co-substrates; nutritional complementation; bioenergy; phenolic compounds; 1G2G ethanol

1. INTRODUCTION

Anaerobic digestion (AD) is an attractive process for managing liquid and solid organic waste that allows energy recovery through biogas, rich in methane (CH_4). Organic matter conversion occurs by the activity of a microbial consortia in a finely-tuned balanced ecosystem. Digested material i.e. digestate can also be exploited as a value-added by-product for agriculture [1]. This biotechnological process is part of the current global context of searching for available residual substrates aligned to the diversification of product generation.

Despite all scientific growth in this area, gaining more knowledge based on innovative issues to comprehensibly investigate interactions between technological and fundamental bioprocess limitations entails optimizing CH_4 generation. For example, the availability of biodegradable fraction in the substrates from the sugar-energy industry (related to AD with consequent CH_4 production) still represents a bottleneck for this scientific field [2]. Insufficient knowledge on principals and operation of AD bioreactors fed with such substrates often results in failed applications in Brazilian sugarcane mills. On the other hand, regarding pre-treatment processes for lignocellulosic biomass to obtain hexose and pentose fractions for other bioprocesses, as in the case of 2G sugarcane ethanol production, enormous advances in fundamental and technological aspects can be found in the literature [3, 4].

Some by-products from the sugarcane agroindustry are already considered raw materials for the recovery and generation of value-added products [5]. Vinasse generated from ethanol distillation is commonly directed to sugarcane culture as liquid-fertile. For each liter of alcohol produced, approximately 10 L of vinasse are generated, and its composition is basically 0.28-0.52 g. L^{-1} of nitrogen (N), 0.11-0.25 g L^{-1} of

phosphorus (P), 1.0-1.4 g L^{-1} of potassium (K) and 20-30 g L^{-1} of Chemical Oxygen Demand (COD) [1, 6]. Sugarcane bagasse, traditionally used in energy generation in Combined Heat and Power (CHP) systems, can be used as a substrate to produce 2G ethanol and other added value by-products [1]. Sugarcane straw, also considered a potential organic source, has become available as a lignocellulosic biomass since the progressive introduction of mechanical harvest without burning procedures in Brazil [7]. In addition to being left in the field for agricultural reasons, straw can be used as feedstock for thermochemical or biochemical conversion processes, which makes it feasible to incorporate it into a biorefinery. Sugarcane straw has chemical composition similar to that of bagasse and can be converted into value-added products and also sugars to produce biofuels, e.g., 2G ethanol, after pre-treatments. Among the diversity of methods that have been researching aiming at technological process improvements, Brenelli et al. [8] recently reported a promissing alkaline pre-treatment of sugarcane straw by deacetylation, in which acetic acid is removed as it is an inhibitor for microorganisms in fermentation processes, and thus, xylo-oligosaccharide (XOS) are recovered for being fermented to ethanol. Filter cake, another organic solid byproduct, is generted from the filtration in rotary filters after cane juice clarification processes, presenting concentrations of 140-169 g kg^{-1} of lignin, 171-184.6 g kg^{-1} of cellulose and 153-170 g kg^{-1} of hemicellulose [2, 9]. It has been used in intrinsic steps at the plant (improvements in permeability during sucrose recovery in the rotary filter) [9] and as a source of nutrients for the soil [10]. Non-controlled digestion of such waste in the fields may lead to the release of large amounts of CH_4, which may hinder the positive effect of bioenergy utilization on climate change mitigation [9].

The economic profitability of biorefineries can be supported by the integrated production of low value biofuels [11]. In this context, co-digestion of residues can optimize CH_4 production by providing and balancing macro and micronutrients for the AD process. It may also be the best option for substrates that are difficult to degrade. This appears to be the case for residues from ethanol production from the processing of lignocellulosic biomass, normally recognized as complex substrates for AD [6]. In addition to intrinsic improvements in the biological process (e.g. upgrading biogas production; better process stabilization by providing synergistic effects within the reactor; increased load of biodegradable organic compounds), the economic advantages of sharing equipment and costs are also successful [12]. Janke et al. [13] showed that co-digestion of filter cake with bagasse would produce 58% more biogas compared to large-scale filter cake mono-digestion. However, there are still gaps in the literature concerning the use of lignocellulosic residues from 2G ethanol production as co-substrates.

Biodegradation capacity of residues can be assessed by Biochemical Methane Potential (BMP) assays. This approach shows the maximum experimental potential to convert the organic fraction of the substrates into CH_4. Specific conditions in AD can also be evaluated: substrate sources (exclusive or blend proportions), temperature, nutrients, buffering, source of inoculum, among other factors. The BMP is the most used methodology by academic and technical practitioners to determine the maximum CH_4 production of a certain substrate [14].

The aim of this paper was to determine the BMP and assess the overall kinetic parameters obtained in AD of sugarcane vinasse, filter cake and deacetylation liquor from the deacetylation pre-treatment of sugarcane straw from the 2G ethanol production

process. Evaluation of BMP and kinetics will take into consideration biogas production of each sole substrate and their codigestion, in different combinations. All experiments were conducted in thermophilic condition since the temperature of crude vinasse leaves the distillation column at 90°C, thus avoiding extra energy expendure to cooling down the bioreactor bulk.

2. METHODOLOGY

2.1 Substrates and inoculum

Vinasse and filter cake from a 1G sugarcane ethanol production process were obtained from Iracema Mill (São Martinho group), Iracemapolis municipality, São Paulo state, Brazil. Deacetilation liquor is as a residue from the biomass pretretreatment to obtain valuable xylooligosaccharides (XOS). Deacetilation liquor was obtained from sugarcane straw in a bench scale 316l stainless steel reactor with 0.5 L capacity. The process was carried out in a at 60 °C with 80 mg NaOH·g biomass^{-1} and 10% (w/w) of final solid loading, immersed in a glycerin bath. Conditions were set based on a previous study to optimize XOS production [8]. The liquid fraction is mainly composed by acetate and phenolic compounds from lignin and extractives and was recovered through straining using a muslin cloth. The deacetylation liquor was then stored at 4ºC for further use.

This study compared two different inocula to perform the anaerobic co-digestion of sugarcane processing residues. In the experiment 1, the inoculum was obtained from a sludge of a mesophilic reactor (BIOPAQ®ICX - Paques) installed at Iracema Mill from São Martinho group (22°35’17.6”S 47°31’51.5”W) treating sugarcane vinasse. Experiment 2 used an anaerobic consortium from athe sludge of a mesophilic Upflow Anaerobic Sludge Blanket (UASB) reactor treating poultry slaughterhouse wastewater

from Ideal slaughterhouse, at Pereiras municipality, São Paulo state, Brazil (23°05'10.5"S 47°58'58.9"W). Adaptation of mesophilic sludge was carried out for avoiding thermal shock to the microbial community. Adaptation consisted in increase the temperature gradually 5 degrees day^{-1} from 30°C until it reaches 55°C [15]. The inoculum was than maintained at this temperature for 1 week prior the beginning of the experiments.

All microorganisms used in this study is registered in the Brazillian Government's system SISGEN (access number A5E04AF), accordingly with Brazillian law for accessing genetic resources and associated traditional knowledge.

2.1 Experimental setup

BMP assays tests were performed their experimental potential for CH_4 production following the protocol stablished by Triolo et al. [16] and VDI 4630 methodology [17]. All experiments were conducted in triplicates of single batches using 250 mL Duran® flasks as bioreactors closet with a pierceble isobutylene isoprene rubber septum, and stored in a XXX incubator kept at thermophilic condition (55°C) and without shacking. Initial pH of each experiment was set at 7.0 through adding solutions of NaOH (0.5 M) or H_2SO_4 (1 M) when necessary. Nitrogen (N_2) gas was fluxed into the liquid medium for 10 min and into the headspace for 5 min after closing the flasks to ensure the assays were performed in anaerobic conditions from their beginning. Headspace was 40% of the volume of the bioreactor. Biogas was collected using a Hamilton® Gastight Super Syringe (1 L) through the flasks' rubber septum. The measured biogas was corrected for a dry gas base by excluding the water vapor content in the wet biogas.

Two rounds of BMP tests were performed. Experiments from both rounds used a 2:1 substrate to microorganism ratio (w/w, in terms of VS). All co-digestion experiments used the substrates in equal proportions. Experiment 1 assessed the sludge from the vinasse treatment plant as inoculum of substrates and the inoculum. Experiment 2 evaluated the BMP using the sludge from the poultry slaughterhouse wastewate treatment plant. In Experiment 2, proportions of inoculum/substrate added in each flask were the same for both rounds of experiments (2:1, w/w). In both experiments, microcrystalline cellulose (Sigma-Aldrich Avicel® PH-101) was used as a positive control (+). Negative control (-) was conducted only with each inocula. Digestion was terminated when the daily production of biogas per batch was less than 1% of the accumulated gas production. Experimental design of both experiments is described in Table 1.

Table 1 – Experimental Biochemical Methane Potential (BMP) design for this study.

Assay		Bulk Composition (%, w/w in terms of VS)				
		Vinasse	Filter Cake	Liquor	Cellulose	Inoculum
Experiment 1	1	33	-	-	-	66
	2	-	33	-	-	66
	3	-	-	33	-	66
	4	11	11	11	-	66
	+	-	-	-	33	66
	-	-	-	-	-	100
Experiment 2	1	33	-	-	-	66
	2	-	33	-	-	66
	3	-	-	33	-	66
	4	17	17	-	-	66
	5	17	-	17	-	66
	6	-	17	17	-	66
	7	11	11	11	-	66
	+	-	-	-	33	66
	-	-	-	-	-	100

2.2 Evaluation of Biochemical Methane Potential

Methane production was evaluated using both the theoretical biochemical methane potential (TBMP) and BMP from the assays. The ratio BMP/TBMP is an indicative of substrate biodegradability and can be considered as an overall efficiency parameter. Volumetric methane production was converted to its normal volume (NL), considering the standard temperature and pressure (STP) conditions (273 K, 1,013 hPa).

Although BMP is traditionally calculated through the average value of the replicates obtained at the end of each batch experiment [17], this method does not consider the trending of the values neither the kinetic model. This work will propose an alternative BMP value obtained through kinetic modelling as the approach suggested by Filer et al. [18], and compares both methods to calculate BMP and biodegradability.

Theoretical Biochemical Methane Potential (TBMP) of filter cake was based on the Buswell equation (Equation 1) for solid substrates. TBMP of deacetylation liquor and vinasse were calculated from their organic matter concentration and volatile solids (VS) content, as depict by (Equation 2) for liquid substrates [14].

$$TBMP_S = \frac{\left[\left(\frac{n}{2} + \frac{a}{8} + \frac{b}{4} + \frac{3c}{8}\right) \cdot 22.4\right]}{12n + a + 16b + 14c} \qquad \text{(Equation 1)}$$

Where $TBMP_S$ is the theoretical biochemical methane potential for solid substrate ($NLCH_4$ $KgVS^{-1}$), 22.4 L mol^{-1} is the molar gas volume in the standard temperature and pressure (STP, 273 K and 1 bar). a, b, c and *n* are the molar content of the substrate for hydrogen, oxygen, nitrogen and carbon, respectively.

$$TBMP_L = \frac{0.35 \cdot OM}{VS} \qquad \text{(Equation 2)}$$

Where $TBMP_L$ is the theoretical biochemical methane potential for liquid substrate ($NLCH_4$ $KgVS^{-1}$), 0.35 NL is the theoretical CH_4 yield of 1 g COD at STP [19], VS is the volatile solids of the substrate (kg L^{-1}) and OM is the organic matter concentration, in terms of COD (gO_2 L^{-1}).

2.3 Kinetics

Co-digestion usually shows a multiple successive methane production arrangement due to the difference between the biodegradability of each substrate. It is expected that the

mixture of substrates leads to a better overall performance than co-digesting each substrate separately. A modified stacked sigmoidal function (Equation 4), based in Boltzmann double sigmoid [20], was used for modelling CH_4 volumetric production in time.

$$V_{CH_4}^{STP}(t) = V_{CH_4}^{max} \cdot \left(\frac{p}{1+e^{\left(\frac{4r_1 \cdot (t_1 - t)}{V_{CH_4}^{max} \cdot p}\right)}} + \frac{1-p}{1+e^{\left(\frac{4r_2 \cdot (t_2 - t)}{V_{CH_4}^{max} \cdot (1-p)}\right)}} \right) \qquad \text{(Equation 4)}$$

Where $V_{CH_4}^{STP}$ is the specific methane production in time (NmL CH_4^{STP} g VS^{-1}), $V_{CH_4}^{max}$ is the maximum specific volumetric production reached in the experiment (NmL CH_4^{STP} g VS^{-1}), p is the proportion between ordinates values of 1st and 2nd stacked sigmoid, t_1 and t_2 are the time which production of the 1st and 2nd sigmoidal pattern reach the maximum rate (d), r_1 and r_2 are the maximum rate of methane production for the 1st and 2nd sigmoidal pattern, respectively (mL CH_4^{STP} g VS^{-1} d^{-1}).

The parameter $V_{CH_4}^{max}$ could be considered as a BMP of the assay since it represents the asymptotic maximum production of methane (when $\lim_{t\to\infty} V_{CH_4}^{STP}(t)$). The main advantage of adopting $V_{CH_4}^{max}$ as the BMP is that it takes in consideration the trending of all experimental data, specially those in the step formed in the end phase of each batch. Thus, this approach to calculate the BMP is more precise than using a simple average of the last values of specific accumulated methane production. Conversely, a lack of any identifiable step in the end of the experiment will severely reduce the goodness of the fiting and increasing the error associated with $V_{CH_4}^{max}$, obliging that its value to be ultimately forced to the value of the simple average of the replicate of the last point.

This model assesses the maximum rate for methane production directly through both r_1 and r_2 parameters. Differently from a classical Boltzmann sigmoidal function, all parameters in this model have a physical meaning. Thus, they could be useful to evaluate the studied process and for further scale-up work based in this present research. All data were processed and fitted using the software Microcal Origin© 2016.

2.4 Physicochemical analysis

2.4.1 Biogas composition

Gas chromatography (Construmaq, São Carlos) analyses were performed to measure the concentration of CH_4. The carrier gas was hydrogen (H_2) gas (30 cm s^{-1}) and the injection volume was 3 mL. Stationary phase was a 3-meter-long stainless steel packed column , diameter of 1/8" with a Molecular Tamper 5A for separation of O_2, N_2 and CH_4. Detection was performed through a thermal conductivity detector (TCD). Equipment was equipped with a specific injector for CH_4, with a temperature of 350°C, an external stainless-steel wall and an internal refractory ceramic wall. Detection limit for CH_4 was 0.1 ppm.

2.4.2 Organic matter

The organic matter content of samples was determined in triplicate according to the Standard Methods for the Examination of Water and Wastewater [21] by the 5220B method for COD determination (digestion and spectrophotometry) and 2540 method for the solid series characterization. The solid series methodology accounted for the concentration of total (TS), volatile (VS) and fixed (FS) solids in the residues characterization.

2.4.3 Sugars and acids

Concentrations of sugars and organic acids were determined in triplicate by High Performance Liquid Chromatography (HPLC, Shimadzu®), composed by pump equipped apparatus (LC-10ADVP), automatic sampler (SIL-20A HT), a CTO- 20A column at 43°C, (SDP-M10 AVP), Aminex HPX-87H column (300 mm, 7.8 mm,

BioRad) and a refractive index detector. The mobile phase was H_2SO_4 (0.01 N) at 0.5 ml min^{-1}.

Furfural and HMF was quantified using a Hewlett-Packard RP-18 column and acetonitrile water (1:8 vv^{-1}) containing 1% (ww^{-1}) acetic acid as eluent in a flow rate of 0.8 mL min^{-1} and a UV detector at 274 nm.

2.4.4 Macro and micronutrient and elementary analysis

An elementary analysis and macro and micronutrient analyses were performed at the Biomass Characterization and Analytical Calibration Resources Laboratory (LRAC), Unicamp. To determine the micronutrients, the substrate samples' ashes were analyzed using the X-ray fluorescence equipment (brand: Panalytical, model: Axios 1KW). The ashes were prepared as is describe in Standard Methods for the Examination of Water and Wastewater [21] for solid series analysis (2540 method). The elementary analysis was possible only for solid samples, i.e., filter cake, by using an elementary carbon, nitrogen, hydrogen and sulfur analyzer (Brand: Elementar, Model: Vari.o MACRO Cube; Hanau, Germany).

2.4.5 Total lignin (phenolic compounds)

Total lignin (soluble + insoluble lignin) content in deacetylation liquor was determined according to [22]. Acid hydrolysis was performed in pressure glass tubes with H_2SO_4 at 4% (w/w) final concentration and autoclaved at 121°C for 1 h. The resulting suspension was filtered and the filtrate was characterized by chromatography to determine concentrations of furan aldehydes (furfural and hydroxymethylfurfural (HMF) – as described in Section 2.4.2).

Insoluble lignin was gravimetrically determined as the solid residue from hydrolysis. For the soluble lignin, an aliquot of the hydrolysate obtained in the acid hydrolysis step was transferred to a flask with distilled water and the final pH was adjusted to 12 with a solution of 6.5 mol L^{-1} NaOH. Soluble lignin was determined from UV absorption at 280 nm using Equation 3.

$$C_{lig} = \frac{(A_{280} \times DF) - (\varepsilon_{HMF} \times C_{HMF} + \varepsilon_{furfural} \times C_{furfural}) - B}{A} \qquad \text{(Equation 3)}$$

Where C_{lig} is the soluble lignin concentration in hydrolysate (g L^{-1}), A_{280} is the absorbance of hydrolysate at 280 nm, DF is the dilution factor, ε_{HMF} is the absorptivity of HMF (114.00 L $g^{-1}cm^{-1}$ – experimental value), $\varepsilon_{furfural}$ is the absorptivity of furfural (146.85 L $g^{-1}cm^{-1}$ – experimental value), C_{HMF} is the HMF concentration in hydrolysate (g L^{-1}), $C_{furfural}$ is the furfural concentration in hydrolysate (g L^{-1}), B is the linear coefficient (0.018 – experimental value), and A is the angular coefficient equal to absorptivity of lignin (23.7 L $g^{-1}cm^{-1}$ – experimental value).

3. RESULTS AND DISCUSSION

3.1 Characterization of substrates

Table 3 shows the general characterization of substrates and inoculum. The COD value of vinasse was within the wide range generally found in the literature (15-35 g O_2 L^{-1}) [1, 6], as well as the VS content (0.015-0.020 g mL^{-1}) [23], while TS content was slightly higher than previously reported (0.020-0.024 g mL^{-1}) [1]. For the filter cake, the TS value was higher than normally reported (literature:0.21-0.28 g mL^{-1}) [2], while VS content was much lower (literature:0.70-0.74 g mL^{-1}) [9]. Such variations reflect the variability of ethanol production processes and the agricultural procedures

affecting biomass characteristics, as well as the sazonality of sugarcane, already stated [1].

Table 2. Main parameter characterization for different substrates and inoculum.

Residue	COD (gO_2 L^{-1})	Volatile Solids (g mL^{-1})	Total Solids (g mL^{-1})	Fixed total Solids (g mL^{-1})	pH	Total Lignin (phenolic compounds) (g L^{-1})
Vinasse [a]	28.81 ± 0.91	0.0184 ± 0.0002	0.0260 ± 0.0063	0.0077 ± 0.0005	4.50 ± 0.35	--
Filter Cake [a]	--	0.2021 ± 0.0005	0.3173 ± 0.0009	0.1152 ± 0.0004	--	--
Deacetylation liquor [a]	32.90 ± 0.27	0.0163 ± 0.0006	0.0215 ± 0.0021	0.0112 ± 0.0001	12.40 ± 0.13	5.50
Iracema Mill Inoculum [a]	12.70 ± 0.42	0.0076 ± 0.0019	0.0154 ± 0.0003	0.0078 ± 0.0000	7.45 ± 0.58	--
Slaughterhouse Inoculum [a]	20.01 ± 0.78	0.0466 ± 0.0076	0.0547 ± 0.0001	0.0081 ± 0.0000	7.32 ± 0.27	--

[a]Three replicates average ± standard deviation; --:not determined

Elementary characterization of filter cake showed that it is mainly composed by 0.16% sulfur, 1.73% nitrogen, 31.56% carbon and 3.11% hydrogen (in %TS). The values for S and N are close to those found in the literature (0.18% and 1.76%, respectively) [24]; however, the C value is below what is normally reported (40-42%) [13]. It resulted in the C:N ratio of the filter cake of 18:1, below what is recommended for AD, which is 20-40: 1 [25].

Slaughterhouse inoculum presented higher values of COD, VS and TS than the inoculum of the sugarcane mill (Table 3), already predicting that it may have a better development for biogas production as it probably contains high cellular mass, i.e. microbiological content. Additionally, slaughterhouse inoculum visually presented a good quality granular appearance from UASB reactors, while the mill inoculum had a liquid aspect. Both pHs were neutral, as expected for anaerobic inocula.

The deacetylation liquor presented a strong alkali characteristic since it came from a mild alkaline pretreatment of sugarcane straw to remove acetyl groups and promote lignin solubilization [8]. Alkaline pretreatment is typically used in lignocellulosic materials such as wheat straw and sugarcane bagasse, thus decreasing its recalcitrance [3]. According to the deacetylation liquor composition (Table 3 and Table 4), a large amount of lignin fractions was detected (phenolic compounds) and high amounts of acids that can be transformed into CH_4, thus showing evidence of a potential high experimental CH_4 production. Several types of pre-treatments are currently carried out with sugarcane lignocellulosic materials, such as chemical (acid, alkaline), biological, physical and physicochemical, in which different types of residues are generated with different characteristics, pH, carbohydrate composition and lignin content [26]. Thus, it is difficult to make comparisons with the literature. It is worth

mentioning that the deacetylation liquor obtained from this work could be specially benefitted for the co-digestion with vinasse due to its basic character. The deacetylation liquor could neutralize the low pH of vinasse without adding large amounts of an alkalizing agent, proving some possible economic benefits of the AD system. The need to alkalize vinasse before AD is an economic disadvantage in terms of implementing this process in sugarcane mills [27]. The presence of C6 and C5 sugars, such as glucose, xylose, arabinose and the presence of oligosaccharides, such as arabionoxylan and glucan (Table 4), is also highlighted which can be used by the anaerobic microbial community for conversion to CH_4, although constraints of AD from C5 sugars are commonly reported [28, 29].

Table 4 describes the main acids and concentrations found in deacetylation liquor and vinasse. High values of acetic acid were obtained for both vinasse deacetylation liquor, in which this volatile fatty acid was reported as important and essential for the acetotrophic methanogenic metabolic route [30]. In addition, Wang et al. [31] noted that concentrations of acetic acid and butyric acid of 2400 and 1800 mg L^{-1}, respectively, did not result in significant inhibition of methanogenics activity. Lactic acid was found in high concentrations in vinasse, and it is usually degraded to propionic acid, which is an undesirable terminal fermentation product; thus high concentrations of propionic acid can result in methanogenesis failure [31]. Moreover, the high concentration of lactic acid in vinasse may result in inhibitory effects for CH_4 production, highlighting the potential advantage of applying the co-digestion to balance the volatile fatty acid composition in the medium. Vinasse also presented malic acid which is generally from the sugarcane plant [31] and isobutyric acid, contributing to its acidic pH.

Table 3. Acid and sugar content of liquid substrates

	Vinasse [a]	Deacetylation liquor [a]
Acetate (mg L^{-1})	2215.91 ± 0.80	3670.00 ± 0.89
Isobutyrate (mg L^{-1})	2076.27 ± 1.50	0.00
Formate (mg L^{-1})	0.00	63.00 ± 1.35
Malate (mg L^{-1})	4944.00 ± 0.48	0.00
Lactate (mg L^{-1})	2618.17 ± 0.98	0.00
Glucose (mg L^{-1})	0.00	85.204 ± 2.45
Glucan (mg L^{-1})	--	626.00 ± 1.12
Fructose (mg L^{-1})	1045.25 ± 0.43	0.00
Arabinose (mg L^{-1})	--	26.00 ± 0.44
Xilose (mg L^{-1})	--	35.00 ± 0.95
Arabionoxylan (mg L^{-1})	--	1747.00 ± 2.32

[a]Mean of three replicates ± standard deviation; --: not determined

Table 5 shows the macro and micronutrient concentrations detected in the substrates. Micronutrients are important for developing AD, mainly because they play a role in the growth of methanogenic microorganisms acting as co-factors in enzymatic reactions [32]. As no external micronutrient solution was added to the experiments, the effects of the nutrient content of the residues could be ascertained by comparing their BMP behaviuour with the positive control test (celullose), which had absence of nutrients. Menon et al. [33] showed optimal concentrations of 303 mg L^{-1} Ca, 777 mg L^{-1} Mg, 7 mg L^{-1} Co and 3 mg L^{-1} Ni that increased biogas productivity by 50% and significantly reduced the processing time. Filter cake presented higher concentrations of the aforementioned micronutrients, except for Ni which was not detected. It is known that an excess of these compounds may cause inhibitory effects on AD, increasing the lag phase of the process [34] or reducing the specific CH_4 production [35]. A considereble amount of S was also detected in filter cake, which could decrease CH_4

formation from acetate due to the sulfate-reducing bacteria activity. Such bacteria compete by using acetate for sulfide production and can even inhibit methanogenesis activity, leading the process to failure [36]. Al and Fe were also present in inhibitory concentrations, which were reported in the literature with values greater than 2.5 g L^{-1} and 5.7 g L^{-1}, respectively [37]. Mg and Ca concentrations were also much above what is recommended for AD (ideally around 0.02 mg L^{-1} and 0.03 mg L^{-1}, respectively), which may also contribute to the inhibition of the process [38]. High concentrations of Mg ions stimulate the production of single cells of microorganisms with high sensitivity for lysis, leading to a loss of acetoclastic activity in anaerobic reactors, while high Ca concentrations can lead to an accumulation of biofilm, which impairs methanogenic activity and may also cause buffering capacity loss of the essential nutrients for AD [37]. On the other hand, cobalt (Co) was detected only in this substrate, within the stimulating concentration range for methanogenesis [39]. These findings reinforce the need of using co-substrates to dilute the potential inhibitory effects caused by excessive concentrations of nutrients in the filter cake, while taking advantage of beneficial effects that certain components of its composition may provide.

Deacetylation liquor presented the main micronutrients in milder concentrations considered important for the development of methanogenic *Archea*, such as Fe, Zn, Cu, Mn, which stimulate reactions catalyzed by metalloenzymes, formation of cytochromes, and ferroxins [40]. However, high concentrations of Si and especially Na were detected. The presence of large amounts of Si is intrinsic of lignocellulosic materials [41]. The use of Si as a trace element for AD is rarely reported, since it is often either volatilized in the biogas produced or else it remains in the digested material [42], not affecting the AD process. The Na can cause an inhibitory effect on the methanization of volatile fatty

acids (mainly propionic acid) in concentrations between 3 to 16 g L^{-1}; however, for glucose rich-substrates, this Na concentration does not significantly affect methanogenesis [43]. Methanogenic archea can also adapt to high Na concentration, leading to high CH_4 conversions [43]. Vinasse did not present known inhibitory concentrations for the assessed macro and micronutrients [37].

Comparing the nutritional content of the inocula, the slaughterhouse inoculum presented a wider range of components in mild concentrations, indicating richer anaerobic microbial activity than the inoculum from the sugarcane mill, especially Co, Ni, Fe content that together allow a better development of methanogenic activity [44]. The mill's inoculum, on the other hand, had neither Co nor Ni trace metals, and much lower Fe concentration. The nutritional poverty of the latter inoculum is accompanied by high K content, consistent with the vinasse treatment, a K-rich substrate.

Table 4. Macro and micronutrient concentration of substrates and inocula

Nutrients	Vinasse (g L^{-1})	Filter Cake (g L^{-1})	Deacetylation liquor (g L^{-1})	Slaughterhouse Inoculum (g L^{-1})	Iracema Mill Inoculum (g L^{-1})
Al	0.0137	16.1825	0.4164	0.3719	0.0402
Ba	0.0000	0.0000	0.0000	0.0014	0.0000
Br	0.0024	0.0000	0.0000	0.0003	0.0013
Ca	0.4682	6.0729	0.4055	0.2349	0.7875
Cl	1.2856	0.0657	0.2546	0.0572	0.7764
Co	0.0000	0.0330	0.0000	0.0023	0.0000
Cr	0.0000	0.0252	0.0000	0.0030	0.0000
Cu	0.0000	0.0230	0.0031	0.1097	0.0016
Fe	0.0163	10.6633	0.3227	1.1316	0.1062
Ga	0.0000	0.0051	0.0008	0.0003	0.0000
Ge	0.0000	0.0000	0.0021	0.0008	0.0000
K	2.6078	0.9487	1.1680	0.2709	1.7843
Mg	0.5372	1.4284	0.1286	0.1155	0.3595

Mn	0.0047	0.2864	0.0123	0.0073	0.0103
Mo	0.0000	0.0000	0.0000	0.0063	0.0004
Na	0.0849	0.0000	10.4902	0.7204	0.0000
Nb	0.0000	0.0040	0.0000	0.0000	0.0000
Ni	0.0000	0.0000	0.0000	0.0028	0.0000
P	0.0913	2.9929	0.1120	0.5496	0.2029
Pb	0.0000	0.0086	0.0000	0.0015	0.0000
Rb	0.0039	0.0042	0.0000	0.0006	0.0030
Si	0.5384	0.5304	1.1620	0.4663	0.0891
S	0.0739	18.1076	0.3345	0.5495	0.3779
Sr	0.0021	0.0419	0.0025	0.0025	0.0033
Ti	0.0013	1.9849	0.0374	0.0200	0.0025
V	0.0000	0.0529	0.0000	0.0000	0.0000
W	0.0000	0.0000	0.0000	0.0033	0.0000
Zn	0.0006	0.0491	0.0043	0.1292	0.0163
Zr	0.0000	0.0537	0.0000	0.0010	0.0000

3.2 BMP: Experiment 1

Time profiles of specific methane production for Experiment 1 are shown in Figure 1, as Table 5 shows the values of each kinetic parameter found in mathematic modeling. Table 6 shows the value of each BMP obtained through the average of the replicates from the assays ending. Co-digestion of substrates enhanced CH_4 prodution when compared to the AD of isolated substrates. However, the positive control (cellulose) did not reach the minimum recommendable BMP value (352 $NLCH_4$ $kgVS^{-1}$) to validate results as maximum potential values for specific CH_4 production [45]. That indicates that maximum capacity for producing CH_4 from the assessed substrates

may not have been reached. The most probable cause for this lack of performance observed in Experiment 1 might be occurred due to the inoculum. Although cellulose digestibility was low, high digestibilities were obtained for liquid substrates (vinasse and deacetylation liquor), which indicates that the presence of nutrients in the substrates (Table 5) has positively affected the inoculum activity as no nutritional supplementation was added in all assays. According Menon et al. [33], the use of micronutrients remedies AD with focus on CH_4 production in thermophilic process and increases biogas productivity. Also, high concentration of acetate in vinasse and deacetylation liquor could be an important factor to boost methane production, since acetate is the sole substrate used by acetoclastic methanogens *Archaea*. In the case of filter cake, despite its high organic content, low biodigestibility was found, probably due to the excess of micronutrients and S concentrations negatively affecting metanogenesis (Table 5) and to physical limitations on the biological process because of its higher TS content (at least 12 times greater than the other co-substrates) (Table 3). The abscence of stirring may have hindered the mass transfer between the substrate and the inoculum, reducing the microbiological reactions involved in the AD process and not allowing to achieve higher BMP values [46].

Fitting of the kinetic model was not adequate for any of the studied substrates in the Experiment 1. As depict in Table 5, cellulose, vinasse, and filter cake must have the $V_{CH_4}^{max}$ value forced to the average of the methane accumulated production at the end of each respective assay so the model could fit. Figure 1 shows no distiguishble step, indicating that those assays might have reached the stationary phase. The absence of this last step in the experimental data leads to a lack of goodness of fiting, although reaching a good coefficient of correlation (R^2). Experiment 1 showed that deacetylation liquor did not show a double sigmoid pattern, indicating that all the methane production occurred in one single step. This same patter

could be observed in the codigestion of vinasse, liquor, and filter cake all together, suggesting that the deacetylation liquor could have improved the process of methane production. Another strong evidence that the deacetylation liquor is the substrate responsible for improving the codigestion is the high value of the apparent kinetic parameter (r_1^{max}) in all experiments with deacetylation liquor, which showed the higher value among the assays performed in the Experiment 1.

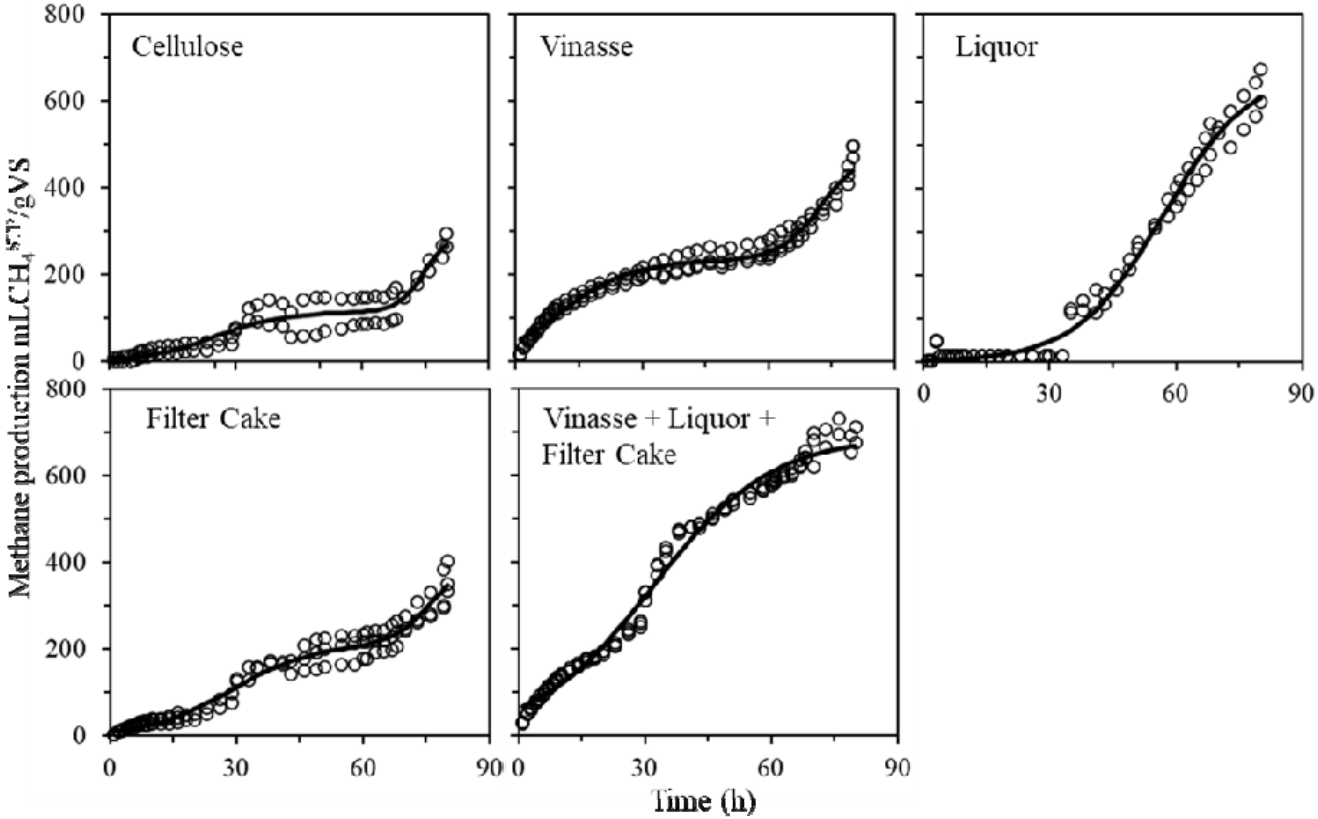

Figure 1 – Time profiles for specific methane production for each substrate and their combination for co-digestion, using sugarcane mill sludge as inoculum. Continuous line depicts the kinetic model fit to experimental data.

Table 5 – Kinetic parameters obtained from time profiles for specific methane production

Substrate	Kinetic Model Parameters						
	$V_{CH_4}^{max}$ (NmL CH_4 gVS^{-1})	p_1	r_1^{max} (NmL CH_4 gVS^{-1} d^{-1})	t_1 (d)	r_2^{max} (NmL CH_4 gVS^{-1} d^{-1})	t_2 (d)	R^2
Cellulose	[a]295.83	0.39 ± 0.02	3.6 ± 0.6	25 ± 2	15 ± 3	75 ± 1	0.89
Vinasse	[a]487.27	0.48 ± 0.01	7.4 ± 0.5	11 ± 1	13 ± 1	72 ± 0	0.97
Liquor	673 ± 20	-	16 ± 0	57 ± 1	-	-	0.99
Filter Cake	[a]404.69	0.53 ± 0.02	5.5 ± 0.4	29 ± 2	11 ± 2	76 ± 1	0.96
Vinasse + Liquor + Filter Cake	688 ± 9	-	12 ± 0	32 ± 1	-	-	0.99

[a]Parameters values forced

Table 6 – Values of experimental BMP, biodigestibility and pH (initial and final) of isolated and co-digested substrates of Experiment 2.

Substrate	TBMP ($NmLCH_4$ gVS^{-1})	[a]BMP ($NmLCH_4$ gVS^{-1})	Biodigestibily (%)		pH	
			[b]Average	[c]Model	Initial	Final
Cellulose	415	282 ± 17	68	71	7.98 ± 0.47	7.69 ± 0.75
Vinasse	548	476 ± 13	87	89	7.13 ± 0.02	7.81 ± 0.15
Liquor	706	610 ± 50	86	95	7.90 ± 0.85	7.80 ± 0.08
Filter Cake	900	362 ± 16	40	45	7.77 ± 0.25	7.83 ± 0.01
Vinasse + Liquor + Filter Cake	718	660 ± 49	92	96	7.91 ± 0.01	7.78 ± 0.47

[a]Average value of the replicates of the last measurement; [b]Calculated considering BMP; [c]Calculated considering $V_{CH_4}^{max}$.

The pH of the assay was adjusted to between 7-8 at the beginning of the experiment and throughout the experiment it remained in this range, occurring neither acidification nor alkalinization.

The accumulated CH_4 volume curves of each test (Figure 1) had standard profiles of AD with a lag phase, exponential growth phase and stationary phase [47]. Despite the high BMP value and high digestibility of deacetylation liquor, its lag phase was significantly long: CH_4 was produced only after 40 days. The long lag phase can be caused by the presence of pre-treatment inhibitors for alcoholic microorganisms, which are commonly reported [48, 49]. However, the presence of furfural or HMF, commonly reported as inhibitors, was not identified. This fact raises two hypotheses: excess of Na, which may have led to a longer time for methanogenic community adaptation (Section 3.1); the presence of fractions of lignin and derived compounds, which may have caused the observed "delay" in the release of organic matter in the environment to access the microbiota. The degradation process of lignin to be used in AD is quite complex, in which some steps are involved before the acetogenesis process [50]. The lignin polymer is first depolymerized and then solubilized, in which different lignin monomers are formed, with varying chain sizes, such as phenylpropanoid derivates with carboxylic acid, alcohol or amine groups. After this stage, these monomers undergo a wide variety of pheripheral pathways to form other intermediates, which are the central monoaromatic intermediate, such as resorcinol (trihydroxibenezene). These elements proceed to the dearometization and cleavage stage of the ring, forming aliphatic acids, which enter the acidogenesis phase and are degraded into volatile fatty acids to continue in the following AD stages [51]. Thus, the long lag phase of deacetylation liquor AD

observed in the BMP test may have happened due to the long process of degradation of lignin fractions and derived compounds, since lignin fractions (i.e., phenolic compounds) were detected in this substrate at significant levels (Table 3).

3.3 BMP: Experiment 2

Table 7 shows the main results from BMP tests of Experiment 2. Unlike Experiment 1, high biodigestibility of cellulose (positive control) was reached (> 85%), thus validating the BMP tests as maximum experimental CH_4 prodution from assessed substrates [45]. The BMP values obtained in Experiment 2 are, thus, the representative ones for the assessed residues.This fact indicates better quality of anaerobic inoculum from the poultry slaughterhouse treatment when compared to the inoculum from sugarcane vinasse treatment. Biogas production constraints from vinasse on a scale (e.g. variation of vinasse composition throughout the season, AD reactor shutdown in the vinasse off-season) reflects the lack of robustness of the inoculum due to its continuous need for adaptation to the substrate, which weakens the microbial activity.

Lower filter cake BMP was obtained when compared to Experiment 1. The physical characteristics of inocula could have played a role in this case: the inoculum from poultry slaughterhouse treatment was composed of very well-formed granules (traditional Upflow Anaerobic Sludge Blanket- UASB sludge), while inoculum from vinasse treatment was liquid without any granules. The mass transfer resistance in anaerobic granules might limit CH_4 production, since the larger the granule, the greater the resistance to mass transfer [52], which may have been attenuated with the liquid inoculum for the filter cake access. Additionally, in the co-digestion BMP tests, the highest value of BMP was obtained with only liquid substrates (deacetylation liquor + vinasse) while using filter cake as co-substrate caused a decrease in BMP values (Table

7). It reinforces that the mass transfer phenomena have an important influence on CH_4 production from filter cake, which must be considered for a reactor operation and inoculum sludge choice. The excess concentrations of some macro and micronutrients already discussed (Section 3.1) may also have contributed to the lower BMP.

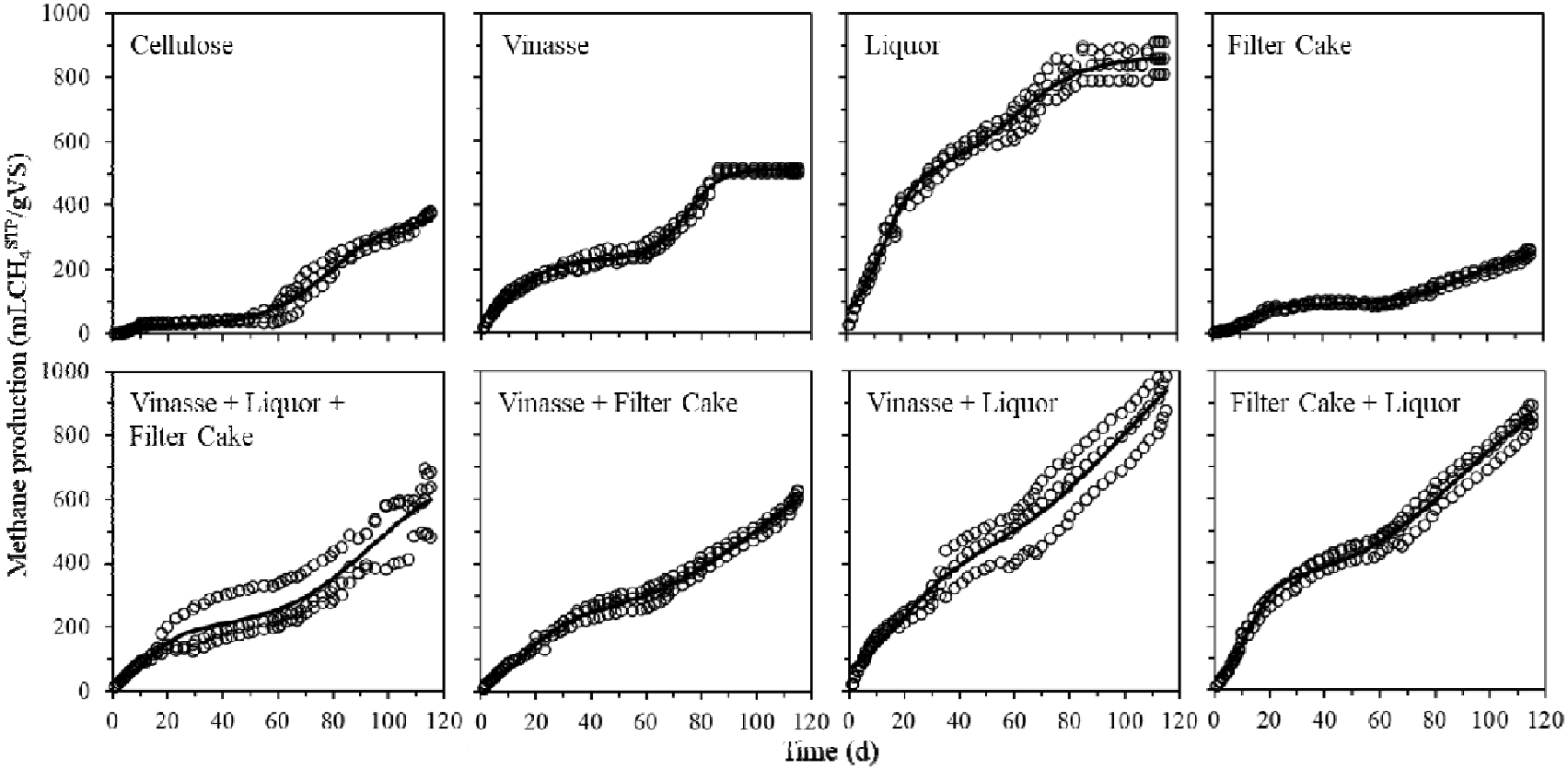

Figure 2 – Time profiles for specific methane production for each substrate and their combination for co-digestion, using poultry slaughterhouse sludge as inoculum.

Table 7 – Kinetic parameters obtained from time profiles for specific methane production.

Substrate	Sigmoid Parameters						
	$V_{CH_4}^{max}$ (NmL CH_4)	p_1	r_1^{max} (NmL CH_4 h^{-1})	t_1 (h)	r_2^{max} (NmL CH_4 h^{-1})	t_2 (h)	R^2
Cellulose	368 ± 7	0.07 ± 0.01	5.6 ± 4.6	7.4 ± 1.1	7.3 ± 0.3	79 ± 1	0.97
Vinasse	513 ± 3	0.44 ± 0.01	7.5 ± 0.4	11 ± 1	11 ± 1	75 ± 0	0.99
Liquor	863 ± 9	0.58 ± 0.03	20 ± 1	12 ± 1	7.3 ± 0.6	62 ± 2	0.98
Filter Cake	274 ± 9	0.32 ± 0.02	5.1 ± 0.4	14 ± 1	3.7 ± 0.1	93 ± 2	0.99
Vinasse + Liquor + Filter Cake	717 ± 119	0.27 ± 0.08	7.2 ± 1.6	13 ± 2	7.7 ± 0.7	94 ± 8	0.90
Filter Cake + Vinasse	797 ± 110	0.28 ± 0.07	7.0 ± 0.4	17 ± 1	6.4 ± 0.2	100 ± 7	0.99
Liquor + Vinasse	1298 ± 660	0.27 ± 0.32	8.3 ± 2.1	18 ± 3	9.1 ± 0.8	102 ± 25	0.95
Filter Cake + Liquor	1021 ± 70	0.31 ± 0.04	17 ± 1	12 ± 0	7.9 ± 0.3	89 ± 4	0.99

Table 8 – Values of experimental BMP, biodigestibility and pH (initial and final) of isolated and co-digested substrates of Experiment 2.

Substrate	TBMP ($NmLCH_4\ gVS^{-1}$)	[a]BMP ($NmLCH_4\ gVS^{-1}$)	Biodigestibily (%)		pH	
			[b]Average	[c]Model	Initial	Final
Cellulose	415	380 ± 7	92	89	7.52 ± 0.45	7.38 ± 0.08
Vinasse	548	507 ± 6	93	94	7.47 ± 0.02	7.12 ± 0.76
Liquor	706	853 ± 43	121	122	7.79 ± 0.15	7.89 ± 0.61
Filter Cake	900	262 ± 2	29	30	7.59 ± 0.89	7.49 ± 0.73
Vinasse + Liquor + Filter Cake	718	605 ± 88	84	100	7.51 ± 2.85	7.35 ± 0.92
Filter Cake + Vinasse	450	614 ± 8	136	177	7.45 ± 1.45	7.32 ± 0.56
Liquor + Vinasse	353	971 ± 72	275	368	7.63 ± 0.39	7.55 ± 0.03
Filter Cake + Liquor	803	861 ± 24	107	127	7.71 ± 0.42	7.87 ± 0.25

[a]Average value of the replicates of the last measurement; [b]Calculated considering BMP; [c]Calculated considering $V_{CH_4}^{max}$.

Experimental BMP of deacetylation liquor showed an atypical result, as it was higher than its TBMP value. Deacetylation pretreatment liquor (with alkaline character) has favorable characteristics for CH_4 production because it reduces the degree of inhibition on CH_4 fermentation [53] , which may explain its high BMP value (Table 7). However, the lower TBMP than BMP implies the possibility that all organic matter in the deacetylation liquor was not accounted for in the COD value, underestimating the value of TBMP. Remnants of insoluble lignin may not have been quantified in the COD analysis [54] and during the BMP tests they may have been hydrolyzed and made available as soluble lignin [51, 54]. CH_4 production from soluble lignin was already reported [55]. It is also worth mentioning that trace metals can act as catalysts favoring the depolarization of the soluble lignin in the liquid medium, thus leaving more organic matter available [56]. The inoculum used in Experiment 1 had lower metal content when compared to the slaughterhouse inoculum of Experiment 2, (especially Al, Co, Fe, Cu) corroborating the hypothesis that the presence of metals may have contributed to the depolarization of soluble lignin in the deacetylation liquor. Thus, larger metal content in poultry inoculum may lead to larger amounts of available organic matter during the BMP test, which was not accounted for in the COD value of deacetylation liquor determined in the absence of inoculum. These assumptions highlight the need for deeper further studies on CH_4 production from liquid lignocellulosic substrates.

Kinetic moddeling performed in all assays of Experiment 2 showed a particularly good fitting. All assays showed a clear ending step at the end of each assay, and the model represented all data without any need of forcing parameters to a value. As

showed in Experiment 1, deacetylation liquor had the higher values for both $V_{CH_4}^{max}$ and r_1^{max}, demonstrating a higher and faster methane production than any other assay. Codigestion of liquor and vinasse and liquor and filter cake have been proved more capable of methane production than other conditions. Kinetic parameters also confirm that codigestion was more effective than single substrate AD for methane production, and the deacetylation liquor was probably the substrate that boosted methanogenesis in co-digestion assays.

As in Experiment 1, the pH of Experiment 2 remained neutral throughout the operation, with no acidification or alkalinization of the medium, and no need for initial pH correction exclusively for the co-digestion test.

As in Experiment 1, the co-digestion of substrates showed a higher potential for CH_4 production than the AD of isolated residues, except for the deacetylation liquor. However, considering the context of a sugarcane biorefinery, its most abundant residue (i.e., vinasse) must be properly managed, whereby AD is an advantageous alternative as already reported [6]. The enhancement of CH_4 production from vinasse can be achieved by adding other residues within the biorefinery boundary as co-substrates, as proved in the current work. By predicting a co-digestion reactor operation, in which the continuous stirred tank reactor (CSTR) is the traditional one [1], the disadvantage of the filter cake by having a higher ST content could be minimized due to stirring, avoiding its sedimentation and improving the substrate-inoculum contact and, therefore, resulting in increased CH_4 production.

Figure 2 shows the curves of cumulative volume of produced CH_4 in Experiment 2, presenting a more accentuated behavior of AD occurring in two-phases when compared to Experiment 1: the acidogenic phase and the subsequent methanogenic phase [57]. This proves that the origin of the inoculum plays an important role in the production of CH_4, as the same substrates were used in the two rounds of experiments. It can be observed that the BMP of the deacetylation liquor had a shorter lag phase when compared to Experiment 1, indicating that there was a better adaptation of the inoculum to the substrate. Gu et al. [58] observed different performance behaviors of biogas production using different inocula for the same substrate (rice straw), showing that some inocula were better adapted to others due to their specific enzymatic arsenal and to the degraded organic matter load: the greater the organic matter converted by the inoculum, the better it would be able to convert lignocellulosic residues. The inoculum used in Experiment 2 came from a consolidated UASB reactor continuously treating

poultry slaughterhouse waste, with higher organic loads fed to the reactor when compared to the inoculum used in Experiment 1 (from a reactor that has been in operation for only 4 years for the treatment of vinasse). This made the slaughterhouse inoculum more robust than mill inoculum, and, thus, more suitable and efficient to convert lignocellulosic materials, causing the smallest lag phase and making the digestion process more stable, which results in higher cumulative CH_4 volumes [59].

4. CONCLUSION

Anaerobic inoculum maturity improved the slow conversion of lignin-fraction monomers into CH_4 from deacetylation liquor. Its alkali-characteristic may contribute to the AD operational costs reduction in an industrial scale as it avoided the reactor alkalizing demand. The highest filter cake TS content indicated operational adjustments needs, e.g. stirring to minimize the mass transfer resistance between substrate-microrganisms. This small-scale study shows how the co-digestion made use of residues positive synergisms to increase CH_4 yield by at least 16%, and is advantageous for the management of the voluminous residue of integrated 1G2G sugarcane biorefineries (vinasse) and those newer and lesser known: the lignin-rich wastes.

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