Biochemical Methane Potential (BMP) from sugarcane biorefinery residues: maximizing their use by co-digestion

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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 of sugarcane vinasse, filter cake and deacetylation liquor from the deacetylation pre-treatment of sugarcane straw from the 2G ethanol production process. The effectiveness of performing co-digestion

of aforementioned residues for optimizing CH_4 production was also assessed, enlarging alternatives for implementing sustainable integrated biorefineries.

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), São Paulo state, Brazil. Deacetylation liquor was obtained from an alkaline deacetylation of sugarcane straw performed on a bench scale. Pretreatment was carried out at 60°C, 80 mg NaOH g biomass^{-1} and 10% (w/w) of final solid loading, in a 316 L stainless steel reactor of 0.5 L capacity, immersed in a glycerin bath. The pretretreatment conditions were obtained based on a previous study to optimize XOS production, whose liquor is considered a residue of this process [8]. The liquid fraction mainly composed by acetate and phenolic compounds from lignin and extractives, referred to as deacetylation liquor, was recovered by straining it through a muslin cloth and stored at 4ºC for further use.

Anaerobic consortium from a mesophilic reactor (BIOPA®CICX - Paques) treating sugarcane vinasse (Iracema Mill, São Martinho group) and an anaerobic consortium from a mesophilic Upflow Anaerobic Sludge Blanket (UASB) reactor from Ideal poultry slaughterhouse (Pereiras, São Paulo state, Brazil) were used in Experiment 1 and Experiment 2 (Section 2.3), respectively.

2.2 Biochemical Methane Potential (BMP) of substrates

Theoretical Biochemical Methane Potential (TBMP) of filter cake was based on the Buswell equation (Equation 1). TBMP of deacetylation liquor and vinasse were

147 calculated from their Chemical Oxygen Demand (COD) and Volatile Solids (VS)
148 content (Equation 2) [14].

149 $$TBMP = \frac{\left[\left(\frac{n}{2}+\frac{a}{8}+\frac{b}{4}+\frac{3c}{8}\right)*22.4\right]}{12n+a+16b+14c}\ NL\ CH_4 kg^{-1}VS \quad \text{(Equation 1)}$$

150 Where *n* represents the carbon content of the sample, *a* the hydrogen content, *b*
151 the oxygen content and *c* the nitrogen content.

152 $$TBMP = \frac{0.35*VS}{DQO\ residue}\ NL\ CH_4 kg^{-1}VS \quad \text{(Equation 2)}$$

153 Where 0.35 L is the theoretical CH_4 yield of 1 g COD at STP [15], VS is the
154 volatile solids of the residue (in kg L^{-1}) and COD is the Chemical Oxygen Demand
155 (COD) of the substrate (g L^{-1}).

156 BMP tests were performed to determine the biodegradability (BMP/TBMP) of
157 crude substrates and their experimental potential for CH_4 production following the
158 protocol of Triolo et al. [16] and the VDI 4630 methodology (2006) [17]. Batch assays
159 (250 mL Duran flasks) were carried out under thermophilic conditions (55°C) as
160 vinasse leaves the distillation columns at 90°C and thus would have lower (or none)
161 energy expenditure for cooling it to mesophilic conditions. As mesophilic sludges were
162 used in thermophilic tests, the previous acclimatation of inocula was carried out for
163 avoiding thermal shock to the microbial community: the temperature was gradually
164 increased every 5 degrees per day until it reached 55°C, as already demonstrated in the
165 literature [18]. On the first day the temperature was increased to 40°C, then to 45°C and
166 in 4 days it had reached 55°C. After reaching this temperature, the inoculum was kept
167 for 1 week at 55°C, then from the beginning of the experiments.The experiments was in
168 triplicate, with 2:1 inoculum to substrate ratio (in terms of VS) added to each flask,
169 thus ensuring excess of inoculum to consume all the organic matter of the substrate and

achieving its maximum experimental CH_4 production. The pH of solution flasks was corrected to neutrality by 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. The headspace was kept in 40%. Biogas was collected from the headspace over the days by using a Gastight Hamilton Super Syringe (1L) 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. The pressure and temperature for one liter of normal (NL) gas were corrected to the standard temperature and pressure (STP) conditions (273 K, 1,013 hPa). Gas chromatography analyses were performed to measure the concentration of CH_4 in the biogas in a gas chromatograph (Construmaq São Carlos). The carrier gas was hydrogen (H_2) gas (30 cm s^{-1}) and the injection volume was 3 mL. The GC Column was made of 3-meter long stainless steel, 1/8 " in diameter and packaged with Molecular Tamper 5A for separation of O_2 and N_2 and CH_4 in the thermal conductivity detector (TCD). It had a specific injector for CH_4, with a temperature of 350°C with an external stainless steel wall and an internal refractory ceramic wall. Detection (resolution) limits are 0.1 ppm for CH_4. BMP of inoculum was determined as the negative control of the experiments. The cellulose (Avicel PH-101 cellulose) BMP was determined as the positive control assay. Digestion was terminated when the daily production of biogas per batch was less than 1% of the accumulated gas production.

2.3 Experimental arrangement

Two rounds of BMP tests were performed. Experiment 1 assessed the inoculum from vinasse treatment (Section 2.1) and equal percentages (in VS terms) of substrates

194 for the co-digestion test. Experiment 2 assessed the inoculum from poultry
195 slaughterhouse waste treatment (Section 2.1) and the co-digestion conditions were
196 expanded. The proportions of inoculum/substrate added in each flask were the same for
197 both rounds of experiments (2:1 in terms of VS), asmentioned in section 2.2. The
198 experimental designs of Experiment 1 and Experiment 2 are described in Table 1 and
199 Table 2, respectively.

Table 1. Experimental Biochemical Methane Potential (BMP) design of Experiment 1.

BMP Assay	Vinasse (%)	Filter Cake (%)	Deacetylation liquor (%)	Cellulose (%)	Mill Inoculum (%)
1	100	0	0	0	0
2	0	100	0	0	0
3	0	0	100	0	0
4	33	33	33	0	0
Positive control	0	0	0	100	0
Negative control	0	0	0	0	100

Table 2. Experimental Biochemical Methane Potential (BMP) design for Experiment 2

BMP Assay	Vinasse (%)	Filter Cake (%)	Deacetylation liquor (%)	Cellulose (%)	Slaughterhouse Inoculum (%)
1	100	0	0	0	0
2	0	100	0	0	0
3	0	0	100	0	0
4	33	33	33	0	0
5	50	50	0	0	0
6	0	50	50	0	0
7	50	0	50	0	0
Positive control	0	0	0	100	0
Negative control	0	0	0	0	100

2.4 Physicochemical analysis

2.4.1 Oganic matter

The organic matter content of samples was determined in triplicate according to the Standard Methods for the Examination of Water and Wastewater [20] 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.

2.4.2 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.

2.4.3 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 [20] 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).

2.4.4 Total lignin (phenolic compounds)

Total lignin (soluble + insoluble lignin) content in deacetylation liquor was determined according to [21]. 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 4.

$$C_{lig} = \frac{(A_{280} \times DF) - (\varepsilon_{HMF} \times C_{HMF} + \varepsilon_{furfural} \times C_{furfural}) - B}{A} \qquad \text{(Equation 4)}$$

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}) [22], 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 3. 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) [23]; 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 [24].

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 [25]. 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 [26]. 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 [27, 28].

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 [29]. In addition, Wang et al. [30] 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 [30]. 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 [30] and isobutyric acid, contributing to its acidic pH.

Table 4. 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 [31]. 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. [32] 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 [33] or reducing the specific CH_4 production [34]. 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 [35]. 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 [36]. 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 [37]. 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 [36]. On the other hand, cobalt (Co) was detected only in this substrate, within the stimulating concentration range for methanogenesis [38]. 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 [39]. However, high concentrations of Si and especially Na were detected. The presence of large amounts of Si is intrinsic of lignocellulosic materials [40]. 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 [41], 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 [42]. Methanogenic archea can also adapt to high Na concentration, leading to high CH_4 conversions [42]. Vinasse did not present known inhibitory concentrations for the assessed macro and micronutrients [36].

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 [43]. 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 5. 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

The main results of BMP tests of Experiment 1 are presented in Table 6 and the respective curves of cumulative volume of produced CH_4 are presented in Figure 1. Co-digestion of substrates has proved to enhance CH_4 prodution when compared to 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 [44]. It suggests that the maximum capacity for producing CH_4 from the assessed substrates may not have been reached. 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 chemical nutritional supplementation was carried out in the tests. According Menon et al. [32], the use of micronutrients remedies AD with focus on CH_4 production in thermophilic process and increases biogas productivity. 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 lack of agitation 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 [45].

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.

Table 6. Values of theoretical and experimental BMP, biodigestibility and pH (initial and final) of isolated and co-digested substrates of Experiment 1.

Substrate	TBMP ($NmLCH_4\ gSV^{-1}$)	BMP [a] ($NmLCH_4\ gSV^{-1}$)	Biodigestibily (%)	pH [a] (initial)	pH [a] (final)
Vinasse	548.05	475.83 ± 12.72	86.82	7.13 ± 0.02	7.81 ± 0.15
Filter Cake	899.99	362.06 ± 15.88	40.22	7.77 ± 0.25	7.83 ± 0.01
Deacetylation liquor	706.44	609.55 ± 49.83	86.28	7.9 ± 0.85	7.8 ± 0.08
Cellulose	415.00	282.32 ± 16.65	68.02	7.98 ± 0.47	7.69 ± 0.75
Co-digestion	--	660.35 ± 49.19	--	7.91 ± 0.01	7.78 ± 0.47

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

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 [46]. 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 [47, 48]. 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 [49]. 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 [50]. 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).

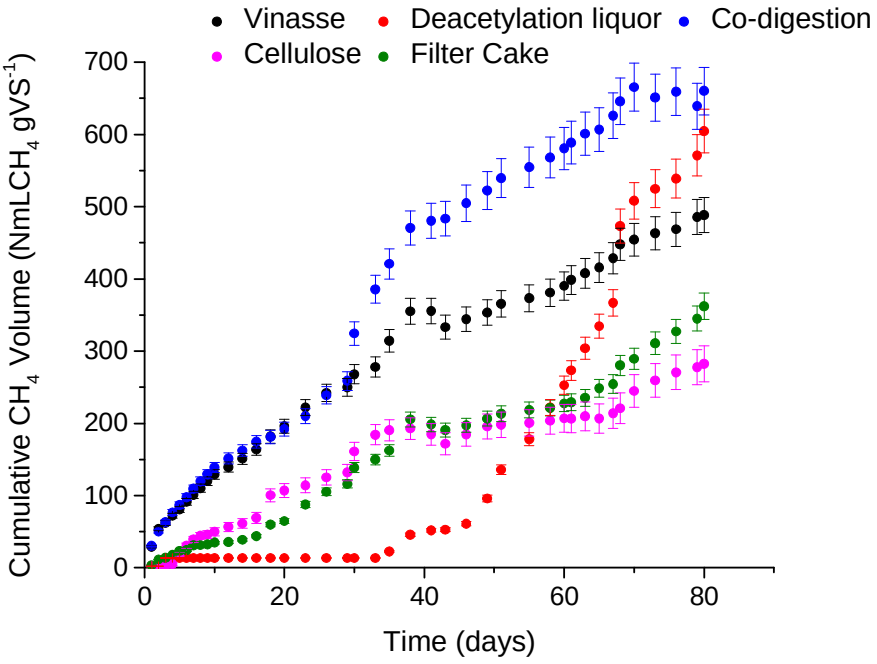

Figure.1 Cumulative methane volume from BMP of Experiment 1

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 [44]. 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 [51], 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.

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 [52] , 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 [53] and during the BMP tests they may have been hydrolyzed and made available as soluble lignin [50, 53]. CH_4 production from soluble lignin was already reported [54]. 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 [55]. 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.

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.

Table 7. Values of experimental BMP, biodigestibility and pH (initial and final) of isolated and co-digested substrates of Experiment 2.

Substrate	BMP [a] ($NmLCH_4\ gSV^{-1}$)	Biodigestibility (%)	pH [a] (inital)	pH [a] (final)
Vinasse	506.76 ± 6.28	92.46	7.47 ± 0.02	7.12 ± 0.76
Deacetylation liquor	852.86 ± 42.68	120.72	7.79 ± 0.15	7.89 ± 0.61
Filter Cake	261.79 ± 1.55	29.08	7.59 ± 0.89	7.49 ± 0.73
Cellulose	380.05 ± 6.70	91.57	7.52 ± 0.45	7.38 ± 0.08
Deacetylation liquor + Filter Cake	861.03 ± 24.06	--	7.71 ± 0.42	7.87 ± 0.25
Deacetylation liquor + Vinasse	970.80 ± 71.55	--	7.63 ± 0.39	7.55 ± 0.03
Vinasse + Filter Cake	614.15 ± 7.75	--	7.45 ± 1.45	7.32 ± 0.56
Vinasse+ Filter Cake + Deacetylation liquor	604.56 ± 88.24	--	7.51 ± 2.85	7.35 ± 0.92

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

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 [56]. 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. [57] 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 [58].

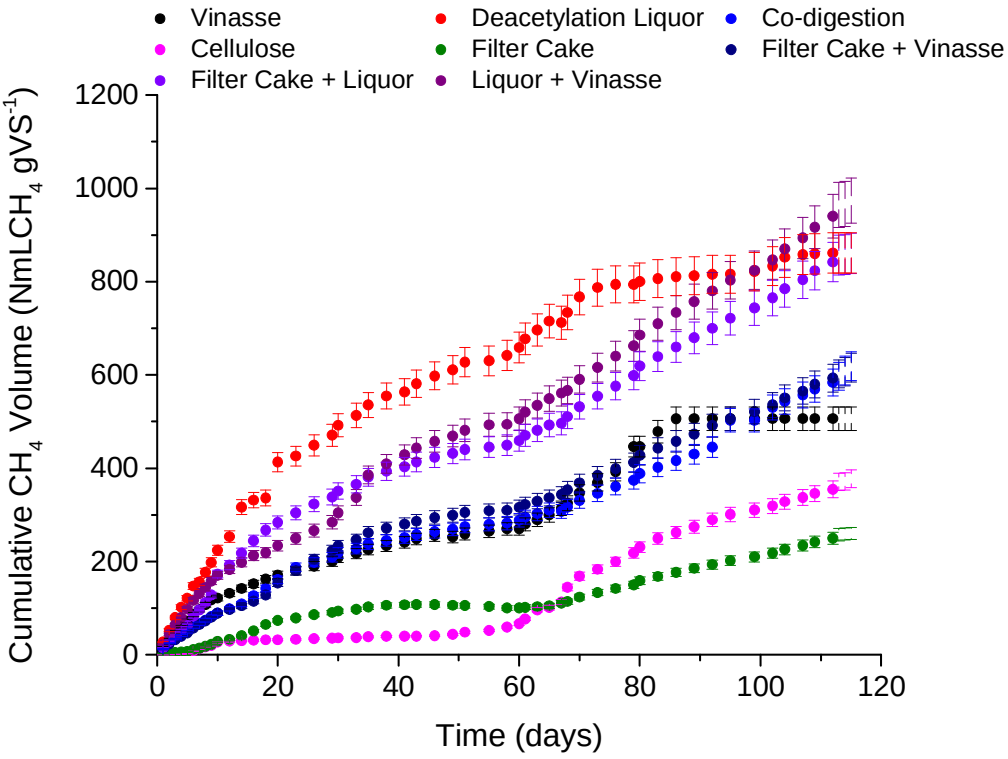

Figure. 2 Cumulative methane volume from BMP of Experiment 2

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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