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Anaerobic Co-Digestion of Vinasse and Pentose Liquor and the Role of Micronutrients in Methane Production within Sugarcane Biorefineries

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Abstract: Anaerobic digestion (AD) of residues from integrated first- and second-generation ethanol (1G2G) biorefineries is a sustainable method for energy recovery through biogas production. This study evaluated the co-digestion of 1G vinasse, 2G vinasse and pentose liquor (from the pretreatment of sugarcane bagasse for 2G ethanol production) compared to individual digestions using biochemical methane potential (BMP) assays. The results showed some "key" micronutrients from the substrates that affected methane (CH_4) production, while their balance provided by co-digestion achieved high digestibility (95%). High iron (Fe) and nickel (Ni) concentrations, in addition to furfural (0.33 g L^{-1}) in pentose liquor seemed to decrease its CH_4 production potential. Despite these adverse effects observed in mono-digestion, co-digestion was beneficial for this substrate, increasing digestibility (52%) and BMP (118%). The highest BMP was observed in vinasse 2G (631 ± 6 NmL CH_4 $gTVS^{-1}$), with no significant difference compared to the adjusted modified Gompertz model (624 ± 10 NmL CH_4 $gTVS^{-1}$). The co-digestion system also presented the highest specific CH_4 production rate (20 ± 1 NmL CH_4 $gTVS^{-1}day^{-1}$) and shortened the lag phase by 19% compared to the AD of isolated 1G vinasse with the second lowest BMP value (494 ± 11 NmL CH_4 $gTVS^{-1}$).

Keywords: biogas; waste-to-energy; biochemical methane potential (BMP)

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1. Introduction

Brazil is the world's largest producer of sugarcane, which is primarily intended for manufacturing first-generation (1G) ethanol and sugar. The estimate for the 2022/2023 harvest made by the National Supply Company (Conab in Portuguese) was 598.3 million tons and the production of sugar and ethanol was 36.4 million tons and 26.6 billion liters, respectively. The main producer and refiner of sugarcane is the state of São Paulo [1].

Technologies to produce lignocellulosic ethanol, the so-called second-generation (2G) ethanol, have been developed through the use of sugarcane bagasse [1,2], which is traditionally used to generate electrical and thermal energy in combined heat and power (CHP) plants. Such technologies basically involve the following steps: (1) the pretreatment step, i.e., to modify the lignin and hemicellulose structures to decrease the polymerization degree and crystallinity of cellulose; (2) the hydrolysis step, i.e., to obtain fermentable sugars from cellulose and hemicelluloses; and (3) the fermentation step, i.e., to convert the fermentable sugars into ethanol. There is interest in developing the 2G process in sugarcane mills

because of the possibility to increase ethanol productivity up to 50% while keeping the same planted sugarcane area [3,4]. However, the Brazilian 2G ethanol industry is still in a consolidation phase and engineering of the mechanical process (which requires significant capital contributions) is the main challenge to overcome [5].

Both 1G and 2G ethanol production processes generate large amounts of residues with high polluting potential. Vinasse from the distillation of ethanol is the most voluminous waste stream generated, ranging from 10 to 15 L of vinasse per liter of ethanol. Its main destination is the fertigation of sugarcane fields due to the appreciable concentrations of calcium (Ca), magnesium (Mg), nitrogen (N), phosphorus (P) and, above all, potassium (K) [6]. Thus, the consumption of fertilizers is reduced; however, excessive addition may cause leaching of ions to deep water or surface runoff and greenhouse gas (GHG) emissions [7], requiring the application of techniques and restrictions for its proper use. The high content of organic matter and salinity, the acid and corrosive character and the presence of phenols and heavy metals are also potential contributors to soil pollution [6].

In the case of 2G ethanol production, attention should be given to the liquid waste from the pretreatment step, with variable compositions depending on the technology process. Physical, chemical, physicochemical and biological treatments or a combination of these processes can be used as a pretreatment [8,9]. Acid pretreatment is the most widely used because of its high yields and cost-effectiveness [10]. The residual liquid (i.e., pentose liquor) is normally composed of sugars (xylose, glucose and arabinose); hemicellulose decomposition products, e.g., oligomers from the polymers and acetate from the hydrolysis of acetyl groups linked to sugars and/or monosaccharides decomposition products, e.g., furfural (product of pentose dehydration); and 5-hydroxymethylfurfural (HMF, product of hexoses dehydration) [3]. The latter may cause inhibition of microbial growth depending on the concentration. However, the organic matter content of pentose liquor suggests it may have the potential to be a substrate for a successful waste-to-energy management process, i.e., anaerobic digestion (AD).

AD can reduce the waste pollutant potential and can recover energy through methane (CH_4)-rich biogas production. The digestate is also an added-value product as a fertilizer that can be applied to culture crops, such as the case of digested vinasse from sugarcane biorefineries [7]. With the development of 2G ethanol production, 2G vinasse and pentose liquor management is a concern as there is little information about these substrates for AD, which has mainly been based on modeling and simulation assessments [11]. In the context of integrated 1G2G sugarcane biorefineries, co-digestion of the aforementioned waste arises as an alternative with some technical advantages inherent to the biological processes, e.g., better nutritional supplementation of the medium by the combination of co-substrates, reduction in potential effects of inhibitory and toxic compounds and enhancement of CH_4 production [12,13].

The digestibility of waste is generally assessed using biochemical methane potential (BMP) assays. These tests measure the maximum experimental potential for converting the organic fraction present in the substrates into CH_4. Initially, a BMP test to evaluate the viability of CH_4 production from a given substrate is a necessary step before implementing AD on a full scale [14]. Recent studies have evaluated the BMP of the residue co-digestion from 1G and 2G sugarcane biorefineries. Volpi et al. analyzed vinasse, filter cake (1G) and deacetylation pretreatment liquor from straw (2G) to produce CH_4, and they discovered that co-digestion of other waste materials with vinasse increased the BMP of mono-digestion of vinasse by at least 16% [15]. Adarme et al. tested 1G vinasse and hemicelluloses hydrolysate (2G), as well as the addition of yeast extract and sugarcane bagasse fly ash in co-digestion, and attained CH_4 yields of 0.279 Nm^3 CH_4/kg chemical oxygen demand (COD), demonstrating that the co-mingling of waste materials improved anaerobic co-digestion and bolstered CH_4 production, thereby furnishing an energy surplus for the process [16]. Wongarmat et al. evaluated sugarcane filter cake, anaerobic sludge and biogas effluent and achieved CH_4 yield productions of 92.8 mL CH_4/g of volatile solids (VS) [17]. These works highlight the benefits of co-digestion in CH_4 production given by

the synergistic effects of improving C/N, nutrient supplementation, alkalinity and trace metals, and reducing toxins or inhibitors.

According to the Brazilian Biogas Association, between 2019 and 2021, there was a 100% growth in production capacity, with the installation of 140 new plants, increasing electricity by 38 MW and biomethane by 120,000 m^3/day [18]. According to the Ten-Year Plan for Energy Expansion prepared by the Energy Research Company (ERC), the production of biogas from vinasse, filter cake and straw and ends could reach 34.9 billion Nm^3 and that of biomethane could reach 19.2 billion Nm^3 by 2032 [19], resulting in a potential of 2.1 GW of electricity generation by 2032. Currently, in Brazil, there are 755 biogas plants in operation, totaling an annual production of 2.3 billion Nm^3 of biogas, with 11% of these plants using industrial effluents and other organic waste from the industrial process [20]. The first large-scale commercial biogas production plant in Brazil, a partnership between Coopcana and Geo Energética, has been in operation since 2012, and the plant uses vinasse, filter cake and straw, with 10 MW of installed capacity [21]. One of the largest biogas plants in the world, a joint venture between Raízen and Geo Energética, has been in operation in the state of São Paulo-Brazil since 2020; the plant used vinasse and filter cake and has an installed capacity of 21 MW [22].

Despite the significant industrial progress in utilizing 1G ethanol production residues for biogas generation and the literature reporting the use of some 2G ethanol production residues, such as straw pretreatment liquors (deacetylation liquor) and pentose liquor (derived from second-generation bagasse pretreatment for ethanol production), there has been limited discourse on the integration of these residues with 2G vinasse itself for biogas generation. In an industrial context, the production of these liquors will consequently generate 2G vinasse, enabling co-digestion of all residues, both from pretreatment and the vinasse itself. However, the challenge of obtaining 2G vinasse has hindered its evaluation in co-digestion processes with pretreatment residues (liquors).

In light of this context, the objective of this work was to investigate the production of CH_4-rich biogas from 1G vinasse, 2G vinasse and pentose liquor generated in 1G and 2G sugarcane ethanol processing. The effects of the substrates in the AD process in a co-digestion system were also investigated. BMP tests and kinetic modeling of experimental data were performed to provide empirical and further conceptual knowledge on co-digestion in the context of an integrated 1G2G sugarcane biorefinery.

2. Results

The physicochemical characterization of the substrates was analyzed based on the organic matter given by the solid series and COD and the levels of volatile organic acids, alcohols and sugars. The values found are shown in Table 1.

Table 1. Physicochemical characterization of the substrates of the BMP tests.

	1G Vinasse	2G Vinasse	Pentose Liquor
pH	5.5	5.0	2.0
Total solids (TS) (g L^{-1})	26.0	33.2	28.3
Total fixed solids (FS) (g L^{-1})	7.7	6.9	2.5
Total volatile solids (TVS) (g L^{-1})	18.4	26.3	25.8
Total suspended solids (TSS) (g L^{-1})	0.4	4.2	0.4
Fixed suspended solids (FSS) (g L^{-1})	0	0.5	0.1
Volatile suspended solids (VSS) (g L^{-1})	0.4	3.7	0.3
COD (g L^{-1})	28.3	48.7	41.5
Glucose (g L^{-1})	1.56	2.91	2.59
Malic acid (g L^{-1})	6.76	0.15	0.21
Acetic acid (g L^{-1})	1.39	1.59	3.68

Table 1. *Cont.*

	1G Vinasse	2G Vinasse	Pentose Liquor
Lactic acid (g L^{-1})	2.10	2.26	0.00
Formic acid (g L^{-1})	0.00	0.00	0.41
Propionic acid (g L^{-1})	1.74	2.85	1.00
Iso-butyric acid (g L^{-1})	0.00	0.80	0.00
Butyric acid (g L^{-1})	5.00	0.00	0.00
Succinic acid (g L^{-1})	1.99	0.60	0.52
Ethanol (g L^{-1})	3.88	10.69	3.48
HMF (g L^{-1})	0.03	0.18	0.02
Furfural (g L^{-1})	0.00	0.00	0.33

The nutritional contents were not similar among the substrates (Table 2). 1G vinasse showed higher concentrations of calcium (Ca), chlorine (Cl) and potassium (K); 2G vinasse had higher contents of sodium (Na) and phosphorus (P), in addition to K; pentose liquor contained higher amounts of aluminum (Al), Ca, Fe, magnesium (Mg), sulfur (S) and K.

Table 2. Concentration of macro- and micronutrients in the substrates.

Micronutrient	1G Vinasse	2G Vinasse	Pentose Liquor
Al (mg L^{-1})	89.7	3.5	917.2
Br (mg L^{-1})	4.6	4.1	N/D
Ca (mg L^{-1})	1711.4	82.2	575.5
Cl (mg L^{-1})	2361.6	N/D	7.1
Co (mg L^{-1})	N/D	N/D	7.2
Cr (mg L^{-1})	N/D	N/D	33.9
Cu (mg L^{-1})	4.2	N/D	1.9
Fe (mg L^{-1})	313.2	8.6	1553.7
K (mg L^{-1})	5868.8	3028	1114.4
Mg (mg L^{-1})	500.7	40.2	275.9
Mn (mg L^{-1})	29.9	1.1	76
Mo (mg L^{-1})	0.9	2.7	N/D
Na (mg L^{-1})	228.2	5537.5	82.6
Ni (mg L^{-1})	2.3	1.6	161.3
P (mg L^{-1})	512.2	2705.3	138.3
Rb (mg L^{-1})	9.7	0.7	2.7
S (mg L^{-1})	N/D	848.7	2237.1
Si (mg L^{-1})	N/D	245.3	572.7
W (mg L^{-1})	N/D	5.7	N/D
Zn (mg L^{-1})	33.1	10.5	6.8

Note: N/D, not detected.

The summary results of the BMP tests are presented in Table 3 and the respective curves of cumulative biogas production can be found in Figure S1. Except for pentose liquor, cumulative biogas production and CH_4 content in biogas were similar among the substrates. The digestibility of the substrate relates the BMP with the theoretical BMP (TBMP) and is also presented in Table 3. The pentose liquor differed from the other substrates, having low digestibility.

The modified Gompertz model adjusted to the experimental data of cumulative CH_4 production for each substrate is presented in Figure 1. The resulting parameters of the model are described in Table 4. It was not possible to fit the pentose liquor experimental data to the modified Gompertz model. An extensive lag phase was observed for the tests, including for microcrystalline cellulose.

Table 3. Summary results of the biochemical methane potential (BMP) tests of the isolated substrates and their co-digestion.

Assay	Cumulative Biogas Production (mL) *	CH_4 Concentration (%) *	TBMP (NmL CH_4 $gTVS^{-1}$)	BMP (NmL CH_4 $gTVS^{-1}$) *	Digestibility *
Cellulose	959 ± 56	53 ± 3	415	395 ± 53	95 ± 13%
1G vinasse	1000 ± 12 [a]	51 ± 8 [a]	538	494 ± 11 [a]	92 ± 2% [a]
2G vinasse	1160 ± 6 [a,b]	55 ± 5 [a]	647	631 ± 6 [a]	98 ± 1% [a]
Pentose liquor	814 ± 34 [a,c]	46 ± 3 [a]	562	256 ± 26 [b]	46 ± 5% [b]
Co-digestion	1020 ± 58 [a,b]	58 ± 8 [a]	588	557 ± 59 [a]	95 ± 10% [a]

* Average ± standard error. Values within a column with the same letter are not significantly different at 5% probability by Tukey.

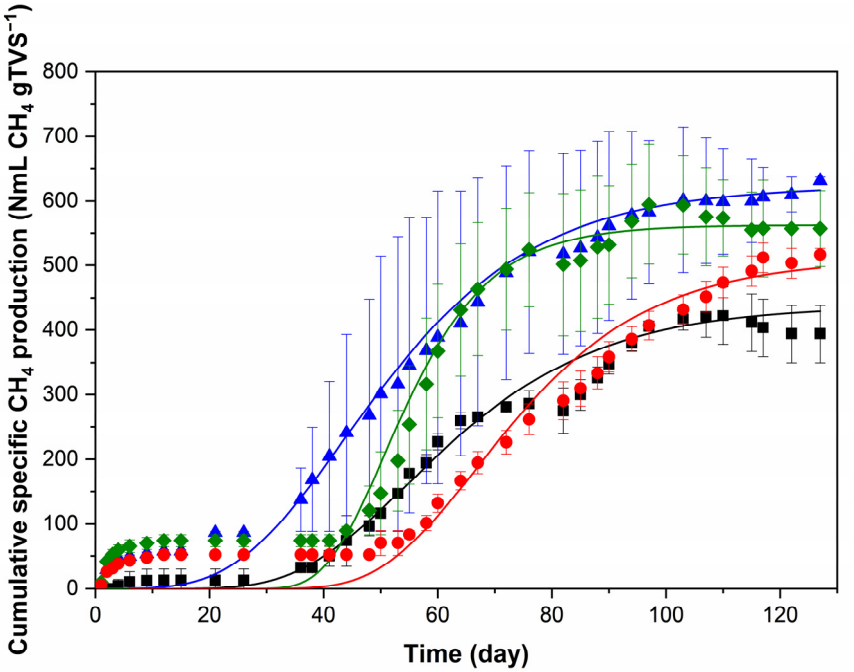


Figure 1. Adjustment to the experimental values of the cumulative CH_4 production of each test under thermophilic conditions (55 °C) to the modified Gompertz model: ■ Microcrystalline cellulose; ● 1G vinasse; ▲ 2G vinasse; ♦ co-substrate.

Table 4. Parameters of the modified Gompertz model found through the nonlinear regression of the OriginPro 2018 software.

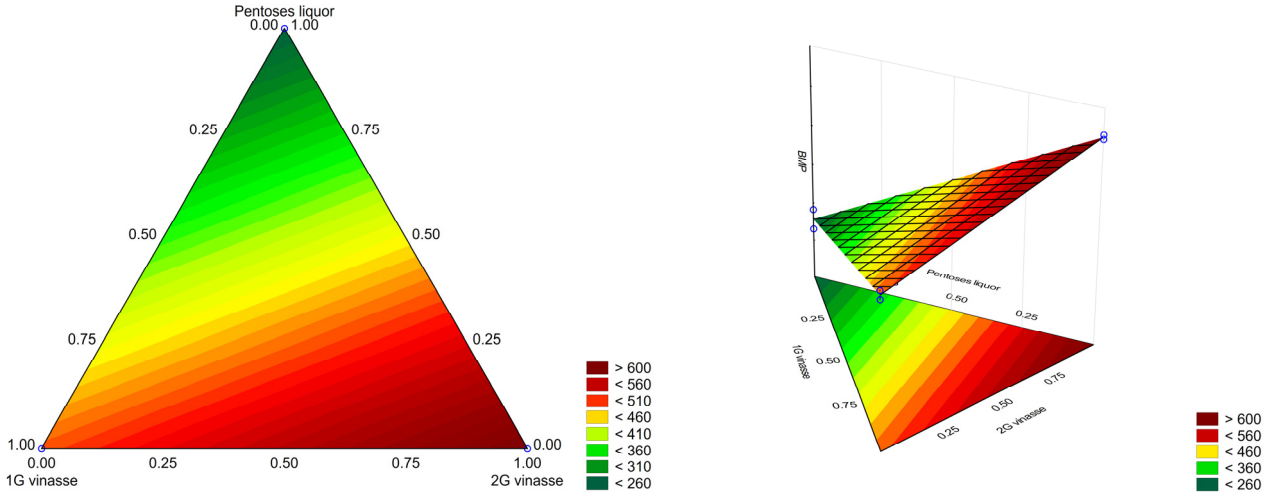
Assay	P (NmL CH_4 $gTVS^{-1}$)	λ (Day)	Rm (NmL CH_4 $gTVS^{-1}$ Day^{-1})	Adj. R^2	Reduced Chi^2
Cellulose [a]	439 ± 10	35.8 ± 0.7	8.5 ± 0.3	0.999	1.273
1G vinasse [a]	510 ± 16	50 ± 1	11.2 ± 0.6	0.998	3.020
2G vinasse [a]	624 ± 10	25 ± 1	12 ± 1	0.998	2.506
Co-digestion [a]	562 ± 12	40.4 ± 0.4	20 ± 1	0.999	0.302

[a] average ± standard error.

The simplified predictive model obtained for representing the correlation of the blend gradient of the co-substrates for specific CH_4 production is presented in Equation (1).

$$BMP = 494\ (\%1GV) + 631\ (\%2GV) + 256\ (\%PL), \quad (1)$$

The mixing gradient can also be evaluated by the triangular surfaces presented in Figure 2.



(**a**) (**b**)

Figure 2. Fitted surface of the blend gradient of the substrates for specific CH_4 production: (**a**) 2D; (**b**) 3D.

3. Discussion

3.1. Characterization of the Inoculum and Substrates

Pentose liquor and 2G vinasse presented the highest levels of TVS, i.e., 25.8 g L^{-1} and 26.3 g L^{-1} (Table 1), respectively, which indicates high potential for biogas production; to a lesser extent, the TVS of 1G vinasse was 18.4 g L^{-1} (Table 1). The COD values followed a similar trend; the COD TVS^{-1} ratios (in the range from 1.5 to 1.9) were indicators of booster substrates to methanogenic activity [23], and thus rich-energy substrates.

The concentrations of TS, TVS and COD (Table 1) in 1G vinasse were similar to those found in the literature [24,25]. Variations reflect the seasonality of this substrate, specificities of the ethanol and sugar production process such as fermentation and yeast used, distillation and wort preparation apparatus and specificities of agricultural practices influencing sugarcane plant composition and, consequently, vinasse composition. In the case of 2G vinasse, the higher values of organic matter (Table 1) were probably due to inefficient ethanol distillation in the rotary evaporator (differently from the industrial distillation columns), allowing residual ethanol in vinasse. However, the results were in the range of the few studies found in the literature, which have reported large variability in 2G vinasse composition [26], since the consolidation of the lignocellulosic ethanol process is still underway. The pentose liquor composition (Table 1) was comparable to that reported from acid pretreatments, in which the organic content is mostly composed of reduced sugars [8,27].

The importance of optimal amounts of nutrients and trace elements for AD has long been recognized [28,29]. Chen et al. [30] reported a stimulant effect of nickel (Ni) and cobalt (Co) on methanogenic activity in concentrations of 5 mg L^{-1}, which highlighted the potential benefit of using pentose liquor (the only one with detected Co) as a substrate for AD. However, excess Ni (161.3 mg L^{-1}) in pentose liquor may be a constraint due to inhibition. Other authors have recommended even lower Ni concentrations, i.e., in the range of 0.6–6 mg L^{-1} [31,32]. All the substrates had a high concentration of Ni, indicating that their dilution may be necessary to improve biogas production. The same has been observed for K concentration, i.e., above 400 mg L^{-1} a toxic effect has been reported [33]. A high concentration of Al can also be a limiting factor for the use of pentose liquor. Cabirol et al. [34] observed a 50% reduction in methanogenic activity specific to a concentration of 1000 mg L^{-1} of Al in the form of $Al(OH)_3$. Leighton and Forster [35] reported that above 1500 mg L^{-1} in the form of $Al_2(SO_4)_3$ there was an inhibitory effect. In addition, the S concentration is much higher than ideal in both pentose liquor and in 2G vinasse, which would be 1–25 mg L^{-1} [36]. The ideal iron (Fe) concentration for AD has been

reported to be in the range of 1–10 mg L^{-1} [32,37], placing 2G vinasse as a potential optimum Fe source. Much higher amounts have been found in 1G vinasse and mainly in pentose liquor; however, Demirel and Scherer [38] reported an optimum Fe concentration in the range of 0.28–50.4 mg L^{-1} specifically for biogas production from agricultural residues. Further investigation on higher Fe concentration in the aforementioned substrates is essential for drawing conclusions about stimulating or inhibitory effects it may cause. Molybdenum (Mo) also acts as an important cofactor with optimum concentration in the range of 0.048–0.35 mg L^{-1} for AD [38,39], which draws attention to possible dilution needed for 1G vinasse and 2G vinasse for improving biogas production. Such adjustments to the nutritional content of substrates for upgrading CH_4 yield might be achieved with the co-digestion process.

3.2. BMP Tests and Digestibility

The largest accumulated biogas volume occurred from 2G vinasse, accordingly with its higher organic matter content. However, the difference in the average volume of accumulated biogas is statistically significant only in the AD of pentose liquor. Pentose liquor showed the lowest biogas production. This difference can be justified by the variation in the composition of the substrates. It is already known that high contents of S (2.23 g L^{-1}), Fe (1.55 g L^{-1}) and Ni (161.3 mg L^{-1}) (Table 2) in pentose liquor can be associated with low production of CH_4 [33,40]. 2G vinasse contained a high concentration of ethanol (10.69 g L^{-1}) (Table 1), a compound easily degraded in AD, which would be in trace concentrations in 2G vinasse obtained in industrial distillation columns. The presence of acids, especially acetic acid (1.59 g L^{-1}) and lactic acid (2.26 g L^{-1}) (Table 1), also favored CH_4 production. The concentrations of acetic acid (1.39 g L^{-1}) and lactic acid (2.10 g L^{-1}) in 1G vinasse were similar to those found in 2G vinasse, apart from a residual ethanol content (3.88 g L^{-1}), compounds known to favor CH_4 production. Co-digestion did not show significant differences statistically in relation to the AD of 1G vinasse and 2G vinasse, both in terms of accumulated volume of biogas and in BMP and digestibility (Table 3). The co-digestion of the substrates, in which the blend presented the highest values of TVS and COD, in addition to a possible complementation of minerals and metals and minimization of potential inhibitors from pentose liquor (which presented the lowest BMP among the substrates), must have contributed to improve methanogenic activity. Such positive effects caused by the co-digestion process have been previously reported with other co-substrate blends [16,41,42].

The digestibility of cellulose, an easily degradable substrate for AD, was in the inferior limit for validating the BMP tests [39]. Its low digestibility indicates nutritional deficiency of the medium for inoculum activity, which is consistent with the extensive lag phase determined by kinetic modeling (Section 3.3). The other substrates, except pentose liquor, had superior BMP and digestibility, especially the co-digestion test, indicating that the nutrients present in the co-substrates relieved the nutritional poverty of the medium. Among the evaluated substrates, the lowest digestibility of pentose liquor might be related to the presence of S, Fe and Ni in the range to generate a toxic response to methanogenic activity [40], being much higher than the reported values for improving AD. Espinosa et al. [43] reported that the addition of Fe (100 mg L^{-1}), Ni (15 mg L^{-1}), Co (10 mg L^{-1}) and Mo (0.2 mg L^{-1}) to the AD of vinasse increased the methanogenic activity from 0.085 to 0.32 g CH_4-COD $gVSS^{-1}$ d^{-1}. Zitomer [44] found that supplementation with 25 mg L^{-1} of Ni, Co and Fe resulted in an increase in the CH_4 production rate to 0.6 of the biomass samples tested. Araujo [45] found an increase in volumetric CH_4 production and in COD removal efficiency from sugarcane vinasse by 101% and 53%, respectively, when concentrations of Fe (76 mg L^{-1}), Ni (0.6 mg L^{-1}) and Co (1.6 mg L^{-1}) were supplemented.

The presence of Cr in pentose liquor may have had a negative effect on CH_4 production as well, since it was much higher than the concentration reported as harmful to the process, i.e., 2 mg L^{-1} [46]. Some potential inhibitors generated from the acid pretreatment such as furfural and derivatives [47] were also detected. The pentose liquor substrate was the only

one containing furfural (0.33 g L^{-1}) (Table 1), with a concentration in the range reported for AD inhibition [48,49]. 5-Hydroxymethylfurfural (HMF), known as another AD inhibitor, was also detected but may not have caused a negative effect on CH_4 production as it was detected in 2G vinasse as well. In addition, the positive nutritional effect of pentose liquor possibly occurred by its Co content within the optimum range referenced in the aforementioned studies. The results confirmed that co-digestion was able to mitigate the inhibitory effects of pentose liquor because of the improvement in the digestibility (52%) and BMP (118%) values compared to the isolated substrate.

2G vinasse had the highest Mo content, and has previously been reported to contribute to the nutritional medium; an optimum concentration of 5 mg L^{-1} (almost twice the value of the present work) has resulted in an increase of 11.6% in the volumetric CH_4 production from food waste [40]. The presence of W, only verified in this substrate, has also been reported to enhance CH_4 production, i.e., 10% improvement was reached from food waste with supplementation of 10 mg of W $kgTS^{-1}$ [50].

The BMP of 1G vinasse (321 NmL CH_4 $gCOD^{-1}$) was higher than some studies have reported under mesophilic conditions. Peixoto et al. [51] obtained 255.44 NmL CH_4 $gCOD^{-1}$ for sugarcane vinasse using nutritional-supplemented medium and carrying out the experiment at 25 °C. Ramos-Vaquerizo et al. [52] reported a BMP value of 244.64 NmL CH_4 $gCOD^{-1}$ for vinasse from sugarcane ethanol production in Mexico at 26 °C. In the case of 2G vinasse, the present work presented a higher value of BMP compared to the very few works found in the literature. Liu et al. [53] reported a BMP value of 272.09 NmL CH_4 $gTVS^{-1}$ of 2G vinasse (at 35 °C) generated in the ethanol production from sugarcane bagasse submitted to simultaneous saccharification and high-fermentation solid production with delayed inoculation.

3.3. Kinetics and Modeling

The modified Gompertz model adjusted to the experimental data of cumulative CH_4 production. The supply of macro- and micronutrients in the medium in synergy with the availability of an easily biodegradable organic compound seemed to be the main factor for shortening the lag phase, which was confirmed by the co-digestion test, which presented shorter lag phase compared to 1G vinasse. This fact illustrates how harmful it is to stop the AD reactor operation during the sugarcane offseason that occurs at the industrial scale for 1G vinasse treatment in most of the Brazilian mills. A long period of consortium acclimatization for effective microbiological activity is needed for the reactor start-up (about 2 months, as shown by our results), which results in considerable loss of CH_4 production, and thus energy generation.

Kinetic data (Table 4) confirmed the observed experimental behavior of CH_4 production from the assessed substrates. Apart from its high CH_4 production potential, the co-digestion test stood out for the highest CH_4 production rate, i.e., at least 67% higher compared to the isolated substrates. The lowest CH_4 production rate from cellulose corroborated the need for nutritional supplementation to improve the methanogenic activity. Similar to the co-digestion test, the 2G vinasse test presented the highest CH_4 production potential but lower CH_4 production rate (Rm), similar to that of 1G vinasse.

The simplified predictive model is suitable for blend gradients, in which the percentages of the substrates are close to the extremes and cannot be used predictively for central blending gradients. It was confirmed that the model is statistically significant ($p < 0.05$) and, notably, 2G vinasse was shown to have the greatest weight to maximize CH_4 production. This conclusion is in line with what was observed in the BMP experiment and in the fitted surface (Figure 2). However, through the statistical analysis, it was found that the three substrates had a significant influence ($p < 0.05$) on the mixing gradient, justifying the application of co-digestion instead of mono-digestion. At the same time, it can be observed that the model did not present a lack of adjustment, in which the sample data were adjusted to the linear model, which can be observed in raw residuals, presented in Figure S2.

4. Materials and Methods

4.1. Inoculum and Substrates

Inoculum and 1G vinasse were collected at the Iracema Mill (Iracemápolis, SP, Brazil) from a high-rate anaerobic bioreactor (BIOPAQ® IC, Paques, SP, Brazil), operated at mesophilic temperature (30 °C) for the treatment of vinasse.

Pentose liquor was obtained from the Pilot Plant for Process Development (PPDP) of the National Biorenewables Laboratory (LNBR), located at the National Center for Energy and Materials Research (CNPEM), Campinas, SP, Brazil. Sugarcane bagasse was subjected to pretreatment with diluted acid in a proportion of 1 kg to 10 L of diluted sulfuric acid (H_2SO_4) solution 5% (*w*/*v*), which was the liquid phase the pentose liquor used in this work. The solid fraction from the pretreatment was washed and subjected to enzymatic hydrolysis with low solids concentration (8% *w*/*v*). The resulting hexose liquor was concentrated up to 80 g glucose L^{-1}, and then was followed by the fermentation step. Then, the obtained wine without yeast (centrifuged) was distilled for producing 2G vinasse. The distillation was performed at the Biocatalysis and Biosynthesis Laboratory of the Chemistry Institute of the University of Campinas (Unicamp, Campinas, SP, Brazil) by using a rotary evaporator (model IKA, RV 10 Control) in batches of 1L heated at 80 °C for 30 min. The production ratio of ethanol to 2G vinasse was 1:13 (L).

4.2. BMP Tests

Before the BMP tests, the mesophilic inoculum was acclimatized to the thermophilic temperature (55 °C) by daily increments of 5 °C, for a period of 1 week. When a temperature of 55 °C was reached, the temperature was kept constant until the end of the experiment. The thermophilic temperature was chosen because the vinasse leaves the distillation columns at 85–90 °C in the industrial process, and thus low or no energy demand is needed for cooling the substrates before feeding the AD reactor [15]. The BMP tests were performed according to the VDI 4630 methodology [54] using 250 mL Duran® flasks in triplicates. A schematic of the experimental setup is shown in Figure 3. The flasks were sealed with a pierceable isobutylene isoprene rubber septum, and then placed in an Ethick-thecnology (411-FPD) incubator set at thermophilic conditions (55 °C). The headspace was kept at 40% of the total volume. The substrate and inoculum ratio in each flask was 1:2 in terms of TVS. The compositions are summarized in Table 5. Tests with microcrystalline cellulose (Avicel PH-101 cellulose) and with only inoculum were performed as positive and negative controls, respectively. The pH in the flasks was adjusted to the range from 7.0 to 7.9 by adding solutions of 50% (g L^{-1}) sodium hydroxide (NaOH). Then, the flasks were flushed with nitrogen gas (N_2) in the liquid medium (3 min) and in the headspace (5 min) after their closure (pressure kept in 5 kgf cm^{-2}) to ensure an anaerobic atmosphere. Three times per week, until the variation of the cumulative CH_4 production remained below 1%, biogas was gathered from the headspace using a gastight Hamilton Super Syringe (1 L) inserted through the rubber septum of the flasks. The wet biogas read-off at room temperature was corrected into dry biogas at standard conditions of temperature and pressure (STP) according to the VDI 4630 methodology [54]. The concentration of CH_4 was analyzed in a gas chromatograph device, CONSTRUMAQ model U-14 (Construmaq São Carlos, SP, Brazil), equipped with a Hayesep D. column and a thermal conductivity detector (TCD). The carrier gas was hydrogen (H_2) at a flow rate of 40 mL min^{-1}, and the injection volume was 3 mL.

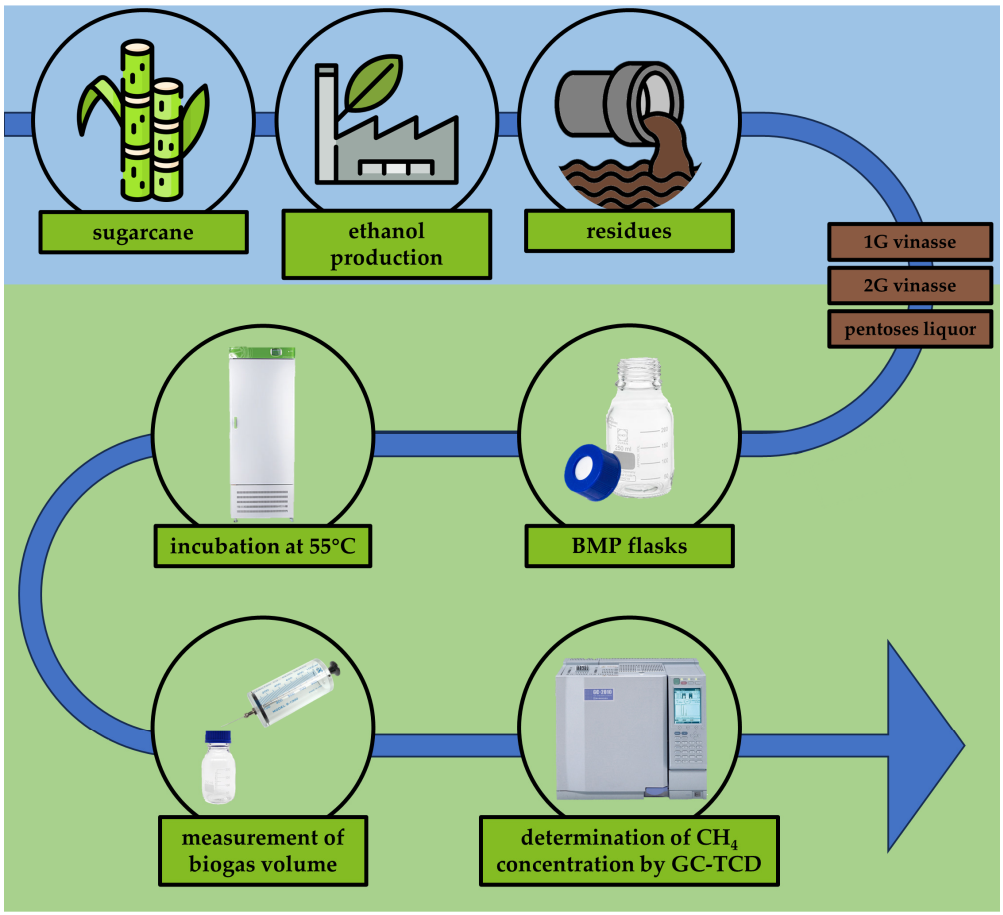


Figure 3. Demonstration of the experimental setup.

Table 5. The compositions of flasks for the BMP tests.

Assay	Assay	Inoculum	Substrate
Negative control	Inoculum	150.00 mL	-
Positive control	Cellulose	148.00 mL	0.49 g
Mono-digestion	1G vinasse	124.30 mL	25.70 mL
Mono-digestion	2G vinasse	131.10 mL	18.90 mL
Mono-digestion	Pentose liquor	130.75 mL	19.25 mL
Co-digestion	1G vinasse	130.00 mL	8.20 mL
	2G vinasse		5.80 mL
	Pentose liquor		6.00 mL

The TBMP of each substrate was determined based on their COD by using the stoichiometric relation of 0.35 NL CH_4 $gCOD^{-1}$ (Equation (2)) and digestibility was calculated using Equation (3):

$$\text{TBMP} = 0.35\ \text{NL}\ CH_4\ gCOD^{-1} \cdot \frac{\text{COD}}{\text{TVS}}, \quad (2)$$

$$\text{digestibility} = \frac{\text{BMP}}{\text{TBMP}}, \quad (3)$$

where TBMP is (NmL CH_4 $gTVS^{-1}$), COD is the chemical oxygen demand (gCOD), and TVS is the total volatile solids (gTVS).

Analysis of variance (ANOVA) was used to identify the existence of significant differences among the substrates, and the Tukey test ($p < 0.05$) was performed to group

data. The analyses were performed using the OriginPro 2018 software (OriginLab Corp., Northampton, MA, USA).

4.3. Physicochemical Methods

The organic matter was determined based on solids series and COD, which were determined according to Standard Methods for the Examination of Water and Wastewater [55] (2540 method and 5220B method, respectively). The absorbance reading of samples was performed on a DR 6000 spectrophotometer (HACH, Loveland, CO, USA). The micronutrient analysis was carried out at the Biomass Characterization, Analytical and Calibration Resources Laboratory (LRAC) at the School of Chemical Engineering at the University of Campinas (UNICAMP). For this analysis, X-ray fluorescence equipment (Malvern Panalytical, model Axios 1kW, Malvern, UK) and a hydraulic press (AMEF, model AP-25T) were used. Volatile organic acids, alcohols and sugars were analyzed by high-performance liquid chromatography using a high-performance liquid chromatograph system (HPLC, Shimadzu Scientific Instruments, Kyoto, Japan equipped with a pump apparatus (LC-10ADVP), an automatic sampler (SIL-20A HT), a CTO- 20A column at 43 °C, (SDP-M10 AVP), an Aminex HPX-87H column (300 mm, 7.8 mm, BioRad, Watford, UK) and a refractive index detector. Quantification of furfural and HMF was conducted in the HPLC as well using a Hewlett-Packard RP-18 column. The eluent, consisting of acetonitrile and water (1:8, vv^{-1}) with 1% (ww^{-1}) acetic acid, flowed at a rate of 0.8 mL min^{-1}. Detection was performed with a UV detector set at 274 nm.

4.4. Modeling of Experimental Data

The modified Gompertz model (Equation (4)) was fitted to the cumulative CH_4 production data of each BMP test.

$$H(t) = P \cdot \exp\left\{ -\exp\left[\frac{R_m \cdot e}{P} \cdot (\lambda - t) + 1 \right] \right\}, \quad (4)$$

where H is the cumulative specific CH_4 production (NmL CH_4 $gTVS^{-1}$), λ is the lag phase time (day), P is the specific CH_4 production potential (NmL CH_4 $gTVS^{-1}$), Rm is the maximum specific CH_4 production rate (NmL CH_4 $gTVS^{-1}$ day^{-1}), t is the incubation time (day), and e is the Euler number (2.71828).

The kinetic parameters were estimated by using the OriginPro 2018 software (OriginLab Corp., Northampton, MA, USA) through nonlinear regression.

The Mix Design statistical package from the STATISTICA 12.5 software was used to estimate a simplified model for predicting CH_4 production according to the proportions of 1G vinasse, 2G vinasse and pentose liquor in the co-digestion process.

Statistical analysis of the predictive model was performed using *p*-value in STATISTICA 12.5. In addition, the triangular surfaces package was used to obtain the fitted surface, as well as a Pareto chart of standardized effects and raw residuals.

5. Conclusions

The highest BMP value was observed for 2G vinasse (624 NmL CH_4 $gTVS^{-1}$), followed by co-digestion (562 NmL CH_4 $gTVS^{-1}$). In contrast, pentose liquor is not a suitable substrate for CH_4 production by mono-digestion. Inhibitory compounds such as furfural and excessive amounts of S, Fe, Ni and Cr hamper the digestion process and reduce digestibility, making it unsuitable for efficient CH_4 production. In general, co-digestion proved to be a suitable alternative for waste management of integrated 1G2G sugarcane biorefineries, enhancing (at least 11%) the specific CH_4 production potential from 1G vinasse, 2G vinasse and pentose liquor, and consequently, improving energy generation. The nutritional supplementation and attenuation of inhibitory effects achieved with co-digestion contributed to superior digestibility of the blend compared to the isolated substrates, indicating improved consortium methanogenic activity. The modified Gompertz model was satisfactorily adjusted to the experimental data, except for the pentose liquor, in which no convergence

was achieved. The highest lag phase of the 1G vinasse AD corroborated the importance of continuous reactor operation throughout the year when considering the industrial scale, as the reactor start-up after the sugarcane offseason would result in considerable delay in achieving the maximum CH_4 potential. The reduction of 10 days in such lag phase obtained by the model with the co-digestion reinforced the advantages of this process. The current study holds significant relevance, especially in light of the increasing contribution of biogas derived from sugarcane biorefinery residues to the Brazilian energy matrix in recent years.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/methane2040029/s1, Figure S1: Cumulative biogas production, Figure S2: Pareto chart of standardized effects and raw residuals.

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