Effect of Biochemical Pretreatment and Nutrient Supplementation on Anaerobic Co-Digestion of Sugarcane Press Mud and Distillery Effluent

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Abstract. Laboratory-scale anaerobic digesters were fabricated and used in the co-digestion of press mud and distillery effluent to investigate the effect of pretreatments (biological/enzymatic and chemical/alkaline) and nutrient supplementation for enhanced biogas production. The findings of this study showed that maximum biogas yield (502.86 mL/g TVS\textsubscript{added}) with maximum percentage of methane (55\%) was obtained in combined enzymatic and alkaline (calcium hydroxide) pretreatment with addition of nutrients (i.e., B6). Combined alkaline pretreatment and nutrient supplementation resulted to a 55.07\% increase in biogas yield compared to the control. Sole nutrient addition enhanced the yield by 64.38\%. However, single alkaline pretreatment presented inhibitory effect which resulted to a 22.48\% decrease in biogas production. Moreover, the percentage of methane gas in the biogas samples was between 14 and 55\%. Samples dosed with enzymes resulted to better methane yields (>50\%) than the samples without enzymes. Highest reductions in BOD, COD and TSS were also achieved in sample B6. Thus, enzymes, especially when combined with other methods, have potential for improving biogas production from sugarcane wastes.

1. Introduction
Over the past century, a vast amount of fossil fuels is being utilized to supply the majority of our energy needs which brought serious concerns such as environmental pollution, climate change and energy resources are consumed much more rapidly. This leads to the search for renewable and eco-friendly alternative energy sources to lessen greenhouse gases emission [1]. One of these renewable energy sources that gained increasing attention among researchers is biogas which can be derived from anaerobic digestion (AD) of organic wastes such as food and kitchen waste, agricultural waste, landfill waste, sewage sludge, etc. [2]. However, because of the insufficient vital trace elements/nutrients and quality of microbial content of one substrate, researchers have explored simultaneous digestion (anaerobic co-digestion) of two or more substrates to surmount the limitations of using mono-substrate, to have better yields and to ensure an economically sustainable biogas production [3,4]. Due to the synergistic effects of two or more substrates with complementary characteristics, co-digestion can lessen toxicity, improve pH buffering capacity and provide better nutrient balance [5]. The substrate’s characteristic is one of the most important factors that govern the biogas yield in AD processes. Thus, different technologies to enhance the biodegradability of substrates and maximize the conversion of organic matter into methane gas in AD are being widely studied. Researchers have also investigated the impacts of nutrient supplementation [6–9]and various pretreatment techniques such as thermal
[10–12], mechanical [13], chemical [14–16], biological [17,18] and combination [19–21] of these methods to ensure a highly efficient biogas production. Sugarcane press mud is a semi-solid by-product/waste from the sugar industry that is obtained from sugarcane juice clarification. Its properties depend on several factors such as the adopted processing of juice clarification, varieties of sugarcane, nutrients used, condition of the soil in the field and other environmental factors. It typically contains ~80% volatile solids that are biodegradable in nature; and its general composition makes it a viable substrate for AD processes [22,23]. However, the hydrophobic and non-degradable lignin content (9-14% on a dry weight basis [24]) in press mud and other fibrous materials prevents the action of microorganisms, making hydrolysis the rate-limiting step during digestion [16]. Among the different pretreatment methods, biological and biochemical treatments were utilized to improve the hydrolysis stage in AD in an eco-friendly way and without using expensive equipment in the process [25]. Distillery effluent is another waste from the bioethanol/sugar industry that can cause environmental concerns when discharged in bodies of water especially with its characteristic unpleasant odor. It also has low pH values and high biochemical oxygen demand (13,414–87,700 mg/L) and chemical oxygen demand (32,000–109,700 mg/L) [26]. Press mud requires water or liquid waste for digestion; hence, distillery effluent can be used as co-substrate since it also has potential in balancing the macronutrients (e.g., C, N, P, K, S and Mg) needed to enhance biogas production, as well as dilute toxic or inhibitory compounds [27]. For instance, López González et al. [24] observed synergistic effects of press mud and vinasse (distillery wastewater) co-digestion due to the better nutritional balance of nitrogen, potassium and sulfur. Moreover, micronutrient supplementation can also be applied to further stimulate methane production since some microelements (e.g., nickel, cobalt, iron, etc.) have been reported vital to the methanogen’s growth and activity in AD processes [6]. Researchers have also explored the addition of microorganisms and enzymes as an alternative to physicochemical pretreatments of substrates [28]; however, direct dosing of enzymes into AD systems has received less attention. Enzymatic pretreatments, especially when combined with other pretreatments, can have great potentials in reducing/breaking down the recalcitrant lignocellulosic structure of sugarcane wastes, yet studies on their effects and efficiency remain limited at present and need further clarification by amassing research on this topic [29]. Not all of the enzymes can improve digestibility since the success of enzymatic pretreatments depends on several factors such as type of enzymes, their functions, stability, dose, etc. [30]. Thus, further testing is needed to optimize this pretreatment strategy for certain types of substrate/waste. To our knowledge, there are no literature studies investigating the effects of biochemical pretreatment, as well as nutrient supplementation, in the codigestion of sugarcane press mud (PM) and treated distillery effluent (DE). Therefore, the aim of this study was to enhance the biogas production from the co-digestion of PM and DE using three techniques: (i) supplementing the biomass mixture with sufficient amounts of macro and micronutrients; (ii) pretreating the substrate with calcium hydroxide in the presence of water at a relatively high temperature and for a certain period of time; and lastly, (iii) stimulation of methanogenic activity by direct dosing of hydrolytic enzymes (i.e., lipases, cellulases, proteases and amylases) into the anaerobic digester.

2. Materials and methods

2.1. Raw materials and inoculum

PM and DE were obtained from Central Azucarera de Tarlac (Tarlac, Philippines) with characteristics listed in table 1. Cow dung was collected from YGGACC HAI Farms (Laguna, Philippines) and digested slops from Absolut Distillers, Inc. (Batangas, Philippines). Prior to their characterization, the samples were stored at 4°C to avoid undesirable fermentation process. The enzyme (Ultrazyme) used in this study is a multicomponent mixture that has a granular powder consistency. It was obtained from Cypher Environmental Ltd. (Winnipeg, Canada) and was stored at room temperature prior to the experiment. According to the manufacturer’s product description, the enzyme mixture was primarily composed of lipases (fat hydrolyzing enzymes), cellulases (cellulose hydrolyzing enzymes), proteases (protein hydrolyzing enzymes) and amylases (starch hydrolyzing enzymes). The inoculum used in the
anaerobic digestion tests was prepared by mixing equal volumes of cow dung and digested slops, which were incubated separately at 37°C for 7 days before starting the experiments.

Table 1. Characteristics and composition of substrates used in this study.

| Parameter                  | PM  | DE            |
|----------------------------|-----|---------------|
| pH                         | 7.56| 4.77          |
| Organic matter (%)         | 15.48| 10.80        |
| Moisture content (%)       | 57.54| NA\(^a\)       |
| Organic carbon (%OC)       | 8.98 | 6.26          |
| Nitrogen (%N)              | 0.95 | 0.26          |
| Total phosphorus (P)       | 7,714 mg/kg | 41.6 mg/L  |
| Carbon-to-nitrogen (C/N) ratio | 9.5  | 24.1          |
| Cobalt (Co)                | < 0 mg/kg | < 0.02 mg/L |
| Copper (Cu)                | 118 mg/kg | < 0.005 mg/L |
| Iron (Fe)                  | 128.57 mg/kg | 2.321 mg/L |
| Magnesium (Mg)             | 242 mg/kg | 15.5 mg/L    |
| Manganese (Mn)             | 2514 mg/kg | 21.5 mg/L   |
| Nickel (Ni)                | 7.58 mg/kg | 0.492 mg/L  |
| Potassium (K)              | 6,530 mg/kg | 228 mg/L    |
| Zinc (Zn)                  | 832 mg/kg | 0.071 mg/L   |

\(^a\) NA, not available or not applicable.

2.2. Pretreatments and nutrient supplementation

For the chemical pretreatment, PM was soaked in low concentration calcium hydroxide solution (62.0 mEq Ca(OH)\(_2\)/L) for 24 hours before heating. DE was diluted in deionized water (50% v/v) before mixing with the alkali-treated PM in a 1:1 ratio. The mixture was heated until boiling for 20 minutes. Then, the substrate mixture was cooled to room temperature before adding the inoculum, enzymes and nutrients. For the biological/enzymatic pretreatment, enzyme (1 g/L) was dissolved in warm water and was injected directly into the digester. For the supplementation of macro and micronutrients, 200 mL of glucose (20 g/L) and 150 mL of a solution containing ammonium chloride (1540 mg/L), monopotassium phosphate (400 mg/L), magnesium chloride hexahydrate (720 mg/L), manganese (II) chloride tetrahydrate (0.027 mg/L), iron (II) sulfate heptahydrate (0.3 mg/L), copper (II) sulfate pentahydrate (2.4 mg/L), cobalt (II) chloride hexahydrate (5 mg/L), zinc sulphate heptahydrate (2 mg/L) and nickel (II) chloride hexahydrate (27 mg/L) were added to the digester. Elemental concentration in the sample after nutrient addition is presented in table 2, including the recommended values according to literature.

Table 2. Elemental composition of feed sample used in this study after nutrient supplementation.

| Elements          | Units | Without nutrients | With nutrients | Recommendations | Reference |
|-------------------|-------|-------------------|---------------|----------------|-----------|
| Phosphorus, P     | mg/L  | 1077              | 1916          | 465            | [31]      |
| Potassium, K      | mg/L  | 1409              | 1830          | < 400          | [28,31]   |
| Cobalt, Co        | mg/L  | 0.7101            | 1.102         | 0.003 – 20     | [9]       |
| Copper, Cu        | mg/L  | 0.562             | 1.288         | 0.03 – 2.4     | [28]      |
| Iron, Fe          | mg/L  | 42.143            | 89.42         | 0.28 – 200     | [9]       |
| Magnesium, Mg     | mg/L  | 174               | 214           | < 720          | [28]      |
| Manganese, Mn     | mg/L  | 18.3              | 22.2          | < 0.027        | [28]      |
| Nickel, Ni        | mg/L  | < 0.02            | 2.9           | 0.029 – 27     | [9,31]    |
| Zinc, Zn          | mg/L  | 2.42              | 2.54          | 0.0327 – 2     | [28,31]   |

2.3. Anaerobic co-digestion

Laboratory-scale digesters were fabricated using 4-L capacity HDPE containers hermetically sealed with rubber stoppers for the co-digestion experiment of PM and DE. Batch samples were prepared
using 500 mL of inoculum, 50 mL enzymes, 350 mL nutrients solution and appropriate amounts of substrate mixture to generate a total working volume of 2500 mL. The experiment was conducted under mesophilic conditions for 30 days with batch sample conditions summarized in table 3. The sample without the nutrients, enzymes and Ca(OH)₂ pretreatment (B1) was treated as control. Nitrogen gas was flushed in each digester for 5 minutes prior to the start of digestion to ensure an anaerobic atmosphere. Biogas volume was measured using 2-L urine bags which were connected to each digester. The volume of gas estimated in the urine bags was taken as the measurement of the biogas produced. All digesters were manually shaken twice a day for 1 minute to ensure efficiency of digestion. Figure 1 shows the experiment procedure.

### Table 3. Experimental conditions.

| Batch test | Substrate mixture | Inoculum | Nutrients | Ca(OH)₂ pretreatment | Enzymatic pretreatment | Nutrient supplementation |
|------------|-------------------|----------|-----------|----------------------|-----------------------|-------------------------|
| B1         | 2000 ml           | 500 ml   | -         | ✖                    | ✖                     | ✖                       |
| B2         | 2000 ml           | 500 ml   | -         | ✔                    | ✖                     | ✖                       |
| B3         | 1650 ml           | 500 ml   | 350 ml    | ✖                    | ✖                     | ✔                       |
| B4         | 1650 ml           | 500 ml   | 350 ml    | ✔                    | ✖                     | ✔                       |
| B5         | 2000 ml           | 500 ml   | -         | ✔                    | ✔                     | ✖                       |
| B6         | 1650 ml           | 500 ml   | 350 ml    | ✔                    | ✔                     | ✔                       |

**Figure 1.** Flow diagram of the 1st phase anaerobic co-digestion experiment.

### 2.4. Daily methane production

The biogas produced from sugarcane residues is typically composed of 50-65% methane (CH₄) and 50-35% carbon dioxide (CO₂) with negligible amount of secondary gases (hydrogen, hydrogen sulfide, ammonia, etc.) [22,24,32]. However, the daily methane generation of the digester can be determined using the liquid displacement method or by absorption of CO₂ in alkaline solution. After the 30-day digestion period of the previously described experiment (first phase), a separate batch test was carried out in a glass reactor using the combination with the highest biogas produced from the first phase to quantify the daily methane production of the reactor for 30 days. The inoculum was reactivated at 37°C for 3 days prior to use. Nitrogen gas was also flushed in the reactor for 5 minutes, sealed with a rubber stopper and mixed manually on a daily basis. The volume of methane was measured by liquid displacement method using an alkaline solution (1% sodium hydroxide) to remove CO₂ [33]. Glass tubing was connected from the reactor to a 2-L cylindrical vessel, where a graduated cylinder was placed to measure the amount of displaced liquid (in mL) as the equivalent of methane gas produced from the reactor bottle. Figure 2 shows the schematic of the daily methane volume collection set-up.
2.5. Analytical methods
Biochemical oxygen demand (BOD), chemical oxygen demand (COD), pH, total organic carbon, total nitrogen, total suspended solids (TSS) and total volatile solids (TVS) of the samples were characterized using standard methods. The methane content of the biogas samples was determined using a gas chromatograph with thermal conductivity detector (GC-TCD). The macro and microelements were analyzed using flame atomic absorption spectroscopy and colorimetry according to standard procedures. The biogas yield (mL/g TVS\textsubscript{added}) after 30 days of digestion was calculated using equation (1) [34]:

$$\text{Biogas yield} = \frac{BV}{TVS\textsubscript{added}}$$

where BV is the cumulative biogas volume (mL) and TVS\textsubscript{added} is the weight (g) of total volatile solids fed to the digester. Also, the removal efficiencies of BOD, COD and TSS were calculated using equation (2):

$$\text{Removal efficiency (\%)} = \frac{C_i - C_f}{C_i} \times 100$$

where $C_i$ and $C_f$ denote the initial and final concentrations of parameters, respectively. Statistical significance of the obtained data was determined by one-way ANOVA (Analysis of Variance) using Microsoft Office Excel with a threshold p-value of 0.05.

3. Results and discussion
3.1. Effect on BOD, COD and TSS
The BOD, COD and TSS values of the digesters before and after AD are presented in table 4. The results showed that the pretreatments and nutrients had no significant effect ($p > 0.05$) on the BOD of the digesters. Similar results were observed for COD and TSS. BOD/COD ratios between 0.3 to 0.8 indicate a high biodegradability rate [35]. The initial BOD/COD of the digesters was in the range of 0.49-0.52 with a mean value of 0.5; this shows that considerable portion of organic matter in the samples is biodegradable in nature. Afterwards, these ratios were reduced to 0.21-0.3 at the end of the AD process.
Table 4. BOD, COD and TSS of digesters before and after 30 days of AD.

| Batch test | Units | Before anaerobic digestion | After anaerobic digestion |
|------------|-------|---------------------------|--------------------------|
|            | BOD   | COD           | TSS          | BOD/COD | BOD   | COD           | TSS          | BOD/COD |
| B1         | 31,133 | 60,004        | 105,917      | 0.52    | 10,850| 46,917        | 24,336       | 0.23    |
| B2         | 31,304 | 62,987        | 67,133       | 0.5     | 9,390 | 42,941        | 17,021       | 0.22    |
| B3         | 31,259 | 63,513        | 18,956       | 0.49    | 9,710 | 45,619        | 8,014        | 0.21    |
| B4         | 30,020 | 60,246        | 42,047       | 0.5     | 11,033| 49,699        | 8,328        | 0.22    |
| B5         | 31,304 | 62,987        | 67,133       | 0.5     | 9,390 | 42,941        | 17,021       | 0.22    |
| B6         | 30,159 | 60,030        | 42,047       | 0.5     | 8,710 | 28,627        | 8,328        | 0.3     |

There were also significant reductions (p < 0.05) in the BOD, COD and TSS values with removal efficiencies (figure 3) of 63-71%, 18-52% and 58-80%, respectively. B6 obtained the highest reductions in all three parameters although its final BOD/COD ratio (i.e., 0.3) suggests that the organic matter in the sample could be further biodegraded. Manyuchi et al. [35] also reported an enhancement in BOD (93%), COD (77%) and TSS (90%) reductions when they used Acti-zyme (bacterial enzyme) as biological pretreatment during the AD of opaque beer wastewater. In addition, another study found that the presence of enzymes, especially lipases and proteases, greatly enhanced the BOD and COD reductions as they can easily break down the organic matter present in the samples [36]. The use of enzymes in AD can therefore increase the rate of biodegradation of the organic and inorganic materials in the samples.

3.2. Effect on pH
Despite the variation on pretreatments and nutrient content, the digesters had no significant difference (p > 0.05) in pH values. At the initial stage of AD, the pH values of the digesters (figure 4) were in the range of 4.14 to 4.86. However, the optimum range of pH that has been reported in literature is 6.5 to 7.6 [37]; the growth of methanogenic bacteria highly reduces beyond this range, thus leading to lower methane yields [38]. Zhai et al. [39] emphasized the significance of controlling the pH at the initial stage of AD to improve methane production. In addition, another study reported an enhancement in reactor performance when the pH was controlled during the first 4 days of the digestion period using sodium hydroxide and hydrochloric acid [40]. It was concluded that the pH adjustment on 7 resulted to
1.87-10.48 times more biogas production from the AD of sugar beet with an average methane content of 36-57%. However, the highest methane yield (72.75%) was achieved when the pH was adjusted to 8. This shows that microbes can also have different pH sensitivity and response to acidic environments. Furthermore, many anaerobic microorganisms remain to be uncharacterized for full understanding of their role in the biogas system. Hence, it is important to conduct more studies about these microorganisms and their functions in order to establish effective operating conditions to achieve maximum biogas performance from sugarcane wastes, as well as to further develop a predictive understanding of the interplay between microbial community structure and operating parameters and performance.

![Figure 4. Initial and final pH values of the digesters.](image)

After the experiment, the pH values were observed to increase (p < 0.05) which is consistent with the work of Zou et al [41]. This increase in pH after the digestion process was due to the conversion of organic nitrogen to ammonium ion (NH₄⁺) [42]. However, the final pH was still not within the recommended range for optimum methanogenic activity. The low pH values of the digesters could be due to the lack of buffering capacity in the substrate and/or excessive accumulation of volatile fatty acids (VFAs) by acidogenic bacteria. Therefore, pH adjustments such as addition of alkali solutions (e.g., sodium hydroxide and calcium carbonate) and proper concentration of total ammonia nitrogen at the initial stage of the process can prevent this problem and neutralize VFAs produced until the system become stable [43]. For instance, Sheng et al. [44] used ammonium chloride as ammonia nitrogen source during the digestion of food waste. It was found that methane production was enhanced by 5.1% when they added 1.1 g/L of ammonia nitrogen compared with the reactors without ammonia nitrogen. While further addition of ammonia nitrogen from 1.9 to 2.82 g/L decreased the methane yield by 7.8% and 18.1%, respectively.

### 3.3. Biogas production

The biogas yield of the digesters is shown in figure 5. The maximum biogas yield was achieved in the digester containing enzymes, nutrients and alkali-pretreated substrate (B6). Based on the figure, the yield increased with the supplementation of macro and micronutrients in the co-digestion of untreated substrates (B3), which was 64.38% higher than the control reactor (B1). However, when the nutrients were added to the alkali-pretreated substrate (B4), the increase in yield was only 55.07%. Researchers
mentioned that the availability of the nutrients for microbial uptake also depends on the system’s pH, temperature, chemical processes, metal speciation, etc. [27]. It is also noteworthy that the sole Ca(OH)$_2$ pretreatment (B2) presented inhibitory effect on biogas production and resulted to a 22.48% decrease in yield. Another aspect that should be taken into account is the possibility of causing secondary pollution from alkaline pretreatments such as the accumulation of large amounts of calcium residues that can have a negative influence on the enzymatic complex accessibility in the biomass during hydrolysis [45]. Researchers often propose Ca(OH)$_2$ as an alternative for alkaline pretreatments because it is relatively cheaper than sodium hydroxide; however, the thermal requirement may not be enough to support the use of Ca(OH)$_2$ instead of NaOH in the process [46]. Monitoring the pretreatment temperature is also important to avoid the production of inhibitory substances. This was also observed from previous studies wherein higher temperatures in alkaline pretreatment decreased the biogas production yield [15]. However, some extreme pretreatment conditions (high pretreatment temperatures) also resulted in an improved AD process for other studies [10]. Therefore, more research on the pretreatment conditions (e.g., stirring, pretreatment/heating time, pressure, solid/liquid ratio, etc.) for this type of waste is suggested.

Despite the Ca(OH)$_2$ pretreatment’s effect on the biogas production, comparing B2 and B5 shows that incorporating biological pretreatment (i.e., enzymes) in the AD of alkali-treated substrate still enhanced the biogas yield by 148.78%; while comparing B5 with control (B1), the increase in yield was 92.86%. This result is consistent with the work of Azman et al. [47] wherein the addition of hydrolytic enzymes counteracted the negative/inhibitory effects of humic acid on the AD of cellulose and xylan in batch reactors. Thus, combined pretreatment methods (i.e., biological and chemical) can lead to better digester performance than single pretreatments during the AD of these wastes. This is because enzymes can remain active even if the conditions of the digester (e.g., salinity, temperature and pH) quickly change, and even in the presence of various toxic and inhibitory substances [28]. Moreover, the biogas from B6 was the highest (502.86 mL/g TVS$_{added}$) among the tests performed. Although more biogas may have been produced if the reaction was performed for a longer period since its BOD/COD ratio after the 30-day digestion period suggests that the organic matter in the sample could still be further biodegraded. Nonetheless, due to the difficulty in degrading tightly lignocellulosic material, enzymes proved to help in breaking down the lignin content in press mud,
which is considered the most important factor affecting the hydrolysis of the cellulose components [48].

Lastly, the difference in biogas yield between B5 and B6 (141.18% higher) shows that the nutrients were also vital for enzymatic activities. In fact, lack of these essential nutrients could affect the function of enzymes and may even lead to digester failure [28]. Muhayodin et al. [49] also mentioned that these trace elements, especially zinc, copper, nickel, cobalt and iron, are important for enzymes involved in the biochemistry of methane formation. Statistical analysis was not performed here because of lacking duplications in data. Table 5 also shows the highest biogas yields derived from other sugar wastes.

Table 5. Biogas production from sugar wastes.

| Waste                        | Operating conditions and pretreatment                                                                 | $Y_{\text{biogas}}$ | % CH$_4$ | Reference |
|------------------------------|------------------------------------------------------------------------------------------------------|---------------------|----------|-----------|
| Press mud and bagasse        | Batch, mesophilic, 30 days; Pretreatment: none                                                      | 455                 | 77.66%   | [50]      |
| Bagasse                      | Batch, mesophilic, 35 days; Pretreatment: hydrothermal and Ca(OH)$_2$                              | 318                 | 69%      | [21]      |
| Sugar beet pulp silage and vinasse | Batch, mesophilic, 30 days; Pretreatment: enzymatic                             | 765.5               | 60.8%    | [51]      |
| Press mud                    | Batch, mesophilic, 30 days; Pretreatment: none                                                      | 241                 | 64.1%    | [22]      |
| Press mud and cane pith      | Batch, mesophilic, 30 days; Pretreatment: none                                                      | 381                 | 66.8%    | [22]      |
| Sugar beet molasses and sewage sludge | Batch, mesophilic, 28 days; Pretreatment: none                                                              | 95.69               | 73%      | [52]      |
| Sugar beet wastes            | Batch, mesophilic, 20 days; Pretreatment: physical (size reduction)                               | 271.26              | 47.25%   | [40]      |
| Press mud and vinasse        | Semi-continuously stirred reactor, mesophilic, 16 weeks; 29.4 days HRT, 1.8 gVS/L/d OLR; Pretreatment: none | 601.7               | 61.2%    | [24]      |
| Concentrated (C$_5$) molasses| Semi-continuously stirred reactor, thermophilic, 70 days; 17 days hydraulic retention time (HRT), 5.3 gVS/L/day total organic loading rate (OLR); Pretreatment: none | 358                 | 59%      | [53]      |
| Press mud and distillery effluent | Batch, mesophilic, 30 days; Pretreatment: enzymatic and Ca(OH)$_2$; Nutrient supplementation | 502.86              | 55%      | This study |

*Biogas yield expressed in mL·gVS$^{-1}$.

3.4. C/N ratio

The carbon-to-nitrogen (C/N) ratio is also a crucial parameter for biogas production; carbon creates the energy source for the microbes while nitrogen results in the formation of ammonia gas. The C/N ratio of the samples is summarized in table 6. Based on the results, the high C/N ratio in B3 and B4 indicates that the samples have excess carbon and/or insufficient nitrogen. Both of these samples were supplemented with macro and micronutrients. This can also explain why these samples have the lowest pH values compared to the others. A study on the AD of sugarcane wastes reported that the required C/N ratio for effective digestion is in the range of 10-30:1 [38,54]. Despite having a C/N ratio higher than the optimum, B3 and B4 still resulted in a better biogas yield compared with the control (D1) that has a 17:1 ratio; this suggests that not all of the carbon in these wastes will also be bioavailable for the microbes due to their high lignin content [27].
Table 6. C/N ratio of the samples.

| Batch test | Ca(OH)₂ pretreatment | Enzymatic pretreatment | Nutrient supplementation | C/N ratio | Biogas yield (mL/g TVS added) |
|------------|----------------------|------------------------|--------------------------|-----------|------------------------------|
| B1         | ✗                    | ✗                      | ✔                        | 17        | 108.11                       |
| B2         | ✔                    | ✗                      | ✗                        | 9         | 83.81                        |
| B3         | ✗                    | ✗                      | ✗                        | 49        | 177.71                       |
| B4         | ✔                    | ✗                      | ✔                        | 32        | 167.65                       |
| B5         | ✔                    | ✔                      | ✗                        | 9         | 208.50                       |
| B6         | ✔                    | ✔                      | ✔                        | 19        | 502.86                       |

Low C/N ratio is also one of the main problems of these wastes for biomethanation. Hence, some alternative options to operate the anaerobic digester at the optimum C/N ratio are by urea supplementation and/or by varying the substrates’ mixing ratio. Aside from balancing the ratio, the addition of urea could also increase the process’ buffer capacity and enhance the digestate’s quality for further use as fertilizers on sugarcane fields [27].

3.5. Methane content

During the digestion period, random biogas samples were tested for methane content analysis, and the results are shown in figure 6. Based on the results, the values of %CH₄ for samples dosed with enzymes were significantly different from the values obtained from the samples without enzymes. Only reactors B5 and B6 achieved a methane content greater than 50%. The low methane yields of the other samples can be attributed to the highly acidic environment in the digester. This supports the claim of Romero-Güiza et al. [28] wherein biological additives/pretreatments (e.g., enzymes) have a better response to stressful digester conditions. Also, these enzymes effectively enhanced the methane production of these sugarcane wastes by penetrating its recalcitrant lignocellulosic structure, thereby causing hydrolysis which is considered the most important factor in the AD of these wastes. These findings were comparative with the study of Wang et al. [29] wherein cellulase and amylase enzymes were both used in improving the hydrolysis and methane yield of corn straw. Meanwhile, another study reported positive effects on methane yield (maximum growth rate of 57.7%) when the same enzymes were used in the AD of sugar beet pulp silage and vinasse.

Figure 6. Methane content of digesters.
On the other hand, lipase enzymes help in catalyzing the hydrolysis of ester linkages in lipids considering that distillery effluents contain 250–6894 mg/L of total lipids [55,56]. Similar results were observed in the study of Prabhudessai et al. [57] where cottage cheese was pretreated with lipases and proteases to enhance the methane production. It was concluded that lipases were very effective in degrading the lipid-rich wastewater. Also, the methane yield was even higher with mixed enzyme pretreatment (1:2 ratio of lipase:protease) compared to single enzyme treatments. As with proteases, these enzymes catalyze the degradation of proteins into their constituent polypeptides and amino acids during hydrolysis. However, in other cases, the addition of proteases to the AD of maize silage, chicken dung and cow manure caused no positive effects probably due to process inhibitions [58]. Thus, not all of the enzymes can improve process digestibility since the success of enzymatic pretreatments depends on several factors such as type of substrates, type of enzymes, their functions, stability, dose, etc. [30]. Overall, the results of this study showed that B6 achieved the highest methane yield (55%). Therefore, hydrolytic enzyme addition is a very great alternative for the enhancement of AD of these wastes, especially when combined with chemical pretreatment methods to destroy lignocellulosic structures, as well as the addition of macro and micronutrients to encourage the multiplication of microbes.

3.6. Daily methane production

For the 2nd phase of the experiment, figure 7 presents the daily and cumulative methane volume from the digester with biochemical pretreatment and nutrient supplementation. The daily methane generation of B6 was further determined to evaluate the energy conversion efficiency of the batch test. As it can be observed from the graph, four peaks appeared during the entire period of the batch experiment (figure 7a). Among these peaks, the highest methane yield was achieved during the 2nd to 3rd day in which the production of daily methane reached 2,845 mL CH₄. Eventually, the enzymes lost their activity in the digester; hence, the yield slowed down at the latter phase of the digestion process because the microbe multiplication also slowed down along with the consumption of organic matter. Moreover, the cumulative methane production enhanced progressively throughout the anaerobic co-digestion phase (figure 7b). Cumulative methane generation of 11,147 mL CH₄ was attained at the end of 30 days. This shows that incorporating appropriate and sufficient amounts of hydrolytic enzymes and nutrients can stimulate methanogenic activity during the co-digestion of alkali-pretreated PM and DE.

![Figure 7. Daily (a) and cumulative (b) methane production of B6.](image-url)
The theoretical methane potential, which could be calculated from the chemical oxygen demand (COD) or elemental composition, is widely used to determine the maximum methane production expected from a substrate [59]. In addition, the biodegradability of the substrate can also be expressed in terms of ratio of the cumulative methane volume (CMV) and theoretical cumulative methane volume (tCMV). The tCMV can be obtained from the ratio of 1 g COD = 0.35 mL CH₄ at STP [37]. Therefore, the tCMV of the sample at 1 atm and 35°C is 60,030 mg COD x (0.395 mL CH₄ /mg COD) = 23,711 mL CH₄. Thus, the CMV/tCMV ratio is 0.5 which indicates that the biomass mixture was relatively biodegradable in nature.

4. Conclusions
Biochemical pretreatment and nutrient supplementation have been successfully used to improve biogas production from the co-digestion of sugarcane press mud and treated distillery effluent. The combined pretreatment methods proved to enhance the methane yield compared with single pretreatment. In this study, the Ca(OH)₂ pretreatment of press mud resulted to a decrease in the biogas yield. One of the disadvantages of this pretreatment is the possible generation of irreversible salts (large amounts of calcium residues) that could be incorporated into the substrate during pretreatment reactions. However, incorporating hydrolytic enzymes (i.e., lipases, cellulases, proteases and amylases) counteracted this inhibitory effect. Biological pretreatment method has many advantages including its no-chemical, low-intensive and mild-condition requirements. Overall, this study showed that enzymatic pretreatment, especially when combined with other methods (e.g., alkaline pretreatment and nutrient supplementation), can stimulate methanogenic activity and improve digester performance (e.g., BOD, COD and TSS reductions) during the anaerobic co-digestion of these sugarcane wastes. However, more research on optimal conditions is also needed.

5. References
[1] Li Y, Chen Y and Wu J 2019 A review Appl. Energy 240 120–37
[2] Hidaka T, Wang F and Tsumori J 2015 Waste Manag. 43 144–51
[3] Matheri A N, Ndweni S N, Belaid M, Muzenda E and Hubert R 2017 Renew. Sustain. Energy Rev. 80 756–64
[4] Søndergaard M M, Fotidis I A, Kovalovszki A and Angelidaki I 2015 Energy & Fuels 29 8088–94
[5] Garcia-Depraect O, Goméz-Romero J, León-Becerril E and López-López A 2017 Int. J. Hydrogen Energy 42 5820–31
[6] Chan P C, Lu Q, de Toledo R A, Gu J-D and Shim H 2019 Sci. Total Environ. 670 337–44
[7] Demirel B and Scherer P 2011 Biomass and Bioenergy 35 992–8
[8] Mancini G, Papirio S, Riccardelli G, Lems P N L and Esposito G 2018 Bioresour. Technol. 247 897–903
[9] Molaey R, Bayrakdar A, Sürmeli R O and Çalli B 2018 Biomass and Bioenergy 108 439–46
[10] López González L M, Reyes I P, Dewulf J, Budde J, Heiermann M and Vervaeren H 2014 Bioresour. Technol. 169 284–90
[11] Barua V B, Rathore V and Kalamdhad A S 2018 Bioresour. Technol. Reports 4 202–8
[12] Ariunbaatar J, Panico A, Yeh D H, Pirrozi F, Lems P N L and Esposito G 2015 Waste Manag. 46 176–81
[13] Tedesco S, Mac D and Ghiani A 2014 Energy 76 857–62
[14] Yuan H, Lan Y, Zhu J, Wachemo A C, Li X and Yu L 2019 Chinese J. Chem. Eng. 27 200–7
[15] Javad M, Karimi K and Sadeghi M 2018 J. Environ. Manage. 226 329–39
[16] Janke L, Leite A, Batista K, Weinrich S, Sträuber H and Nikolausz M 2016 Bioresour. Technol. 199 235–44
[17] Rouches E, Escudié R, Latrille E and Carrère H 2019 Waste Manag. 85 464–76
[18] Zhang Y, Kang X, Wang Z, Kong X, Li L, Sun Y, Zhu S, Feng S, Luo X and Lv P 2018 Energy 164 400–7
[19] Rani R U, Kumar S A, Kaliappan S, Yeom I and Banu J R 2012 Bioresour. Technol. 103 415–24
[20] Si M, Liu D, Liu M, Yan X, Gao C, Chai L and Shi Y 2019 *Bioresour. Technol.* **272** 275–80
[21] Mustafa A M, Li H, Radwan A A, Sheng K and Chen X 2018 *Bioresour. Technol.* **259** 54–60
[22] Rouf M A, Bajpai P K and Joshi C K 2010 *Bangladesh J. Sci. Ind. Res.* **45** 371–6
[23] Radjaram B and Saravanane R 2011 *Biomass and Bioenergy* **35** 2721–8
[24] López González L M, Reyes I P and Romero O R 2017 *Waste Manag.* **68** 139–45
[25] Agabo-garcía C, Rodríguez-morgado B and Parrado J 2019 *Fuel* **255** 115713
[26] Devia-orjuela J S, Alvarezz-puglesi C E, Donneys-victoria D, Cabrales N M, Edith L, Ho B, Br B, Sauciuc A and Briceno M 2019 *Energies* **12** 4145
[27] Janke L, Leite A, Nikolaus M, Schmidt T and Liebetrau J 2015 *Int. J. Mol. Sci.* **16** 20685–703
[28] Romero-Guiza M S, Vila J, Mata-Alvarez J, Chimenos J M and Astals S 2016 *Renew. Sustain. Energy Rev.* **58** 1486–99
[29] Wang X, Cheng S, Li Z, Men Y and Wu J 2020 *Sustain.* **12** 5453
[30] Bonilla S, Choolaei Z, Meyer T, Edwards E A, Yakunin A F and Allen D G 2018 *Biotechnol. Reports* **17** 77–85
[31] Mao C, Feng Y, Wang X and Ren G 2015 *Renew. Sustain. Energy Rev.* **45** 540–55
[32] Mehariya S, Kumar A, Karthikeyan P, Punniyakotti E and Wong J W C 2018 *Bioresour. Technol.* **265** 519–31
[33] Pakarinen A, Maijala P, Jaakkola S, Stoddard F L, Kymäläinen M and Viikari L 2011 *Biotechnol. Biofuels* **4** 20
[34] Srivichai P and Chavalparit O 2020 *Int. J. GEOMATE* **18** 148–55
[35] Manyuchi M M, Mbohwa C and Muzenda E 2018 *South African J. Chem. Eng.* **26** 74–9
[36] Filer J, Ding H H and Chang S 2019 *Water* **11** 921
[37] Matheri A N, Sethunya V L, Belaid M and Muzenda E 2018 *Renew. Sustain. Energy Rev.* **81** 2328–34
[38] Sheng K, Chen X, Pan J, Kloss R, Wei Y and Ying Y 2013 *Biosyst. Eng.* **116** 205–12
[39] Yu Q, Liu R, Li K and Ma R 2019 *Renew. Sustain. Energy Rev.* **107** 51–8
[40] Meléndez-Hernández P A, Hernández-Beltrán J U, Hernández-Guzmán A, Morales-Rodríguez R, Torres-Guzmán J C and Hernández-Escoto H 2019 Biomass Convers. Biorefinery https://doi.org/10.1007/s13399-019-00574-3
[41] Azman S, Khadem A F, Plugge C M, Stams A J M, Bec S and Zeeman G 2017 *Appl. Microbiol. Biotechnol.* **101** 889–901
[42] Domingues R F, Sanches T, Silva G S, Bueno B E, Ribeiro R, Kamimura E S, Franzolin Neto R and Tommaso G 2015 *Food Res. Int.* **73** 26–30
[57] Prabhudessai V, Salgaonkar B, Braganca J and Mutnuri S 2014 Pretreatment of cottage cheese to enhance biogas production Biomed Res. Int. 2014

[58] Müller L, Kretzschmar J, Pröter J, Liebetrau J, Nelles M and Scholwin F 2016 Bioresour. Technol. 203 267–71

[59] Nielfa A, Cano R and Fdz-Polanco M 2015 Biotechnol. Reports 5 14–21