Co-generation of biohydrogen and biochemicals from co-digestion of Chlorella sp. biomass hydrolysate with sugarcane leaf hydrolysate in an integrated circular biorefinery concept

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Abstract

Background: A platform for the utilization of the Chlorella sp. biomass and sugarcane leaves to produce multiple products (biorefinery concept) including hydrogen, methane, polyhydroxyalkanoates (PHAs), lipid, and soil supplement with the goal to achieve the zero waste generation (circular economy) is demonstrated in this study. Microalgal biomass were hydrolyzed by mixed enzymes while sugarcane leaves were pretreated with alkali followed by enzyme. Hydrolysates were used to produce hydrogen and the hydrogenic effluent was used to produce multi-products. Solid residues at the end of hydrogen fermentation and the remaining acidified slurries from methane production were evaluated for the compost properties.

Results: The maximum hydrogen yield of 207.65 mL-H₂/g-volatile solid (VS)added was obtained from 0.92, 15.27, and 3.82 g-VS/L of Chlorella sp. biomass hydrolysate, sugarcane leaf hydrolysate, and anaerobic sludge, respectively. Hydrogenic effluent produced 321.1 mL/g-VS of methane yield, 2.01 g/L PHAs concentration, and 0.20 g/L of lipid concentration. Solid residues and the acidified slurries at the end of the hydrogen and methane production process were proved to have compost properties.

Conclusion: Hydrogen production followed by methane, PHA and lipid productions is a successful integrated circular biorefinery platform to efficiently utilize the hydrolysates of Chlorella sp. biomass and sugarcane leaf. The potential use of the solid residues at the end of hydrogen fermentation and the remaining acidified slurries from methane production as soil supplements demonstrates the zero waste concept. The approach revealed in this study provides a foundation for the optimal use of feedstock, resulting in zero waste.

Keywords: Anaerobic digestion, Polyhydroxyalkanoates (PHAs), Lipid, Soil supplement, Microalgae
Background

Biohydrogen is a renewable energy resource that has received attention because of its sustainability, clean combustion product, and high energy content of 122 kJ/g, which is 2.5 times that of fossil fuels [1]. Biohydrogen is widely produced by dark fermentation due to ease of operation and low operation cost, a high production rate, and commercialization potential [2]. Recently, microalgal biomass has been used to produce biohydrogen by dark fermentation [3]. Microalgae has high protein and carbohydrate content and low lignin levels, However, the low carbon-to-nitrogen (C/N) ratio causes ammonia inhibition [4, 5], resulting in low hydrogen productivity. Thus, co-digestion with a carbon-rich substrate under a suitable C/N ratio is required to eliminate this drawback. Co-digestion involves the use of mixtures of substrates. Its advantages include dilution of toxic compounds, adjustment of the C/N ratio, improved nutrient balance, enhanced synergistic reactions of microorganisms, and increased fermentation efficiency [5, 6]. In the present study, microalgal biomass was co-digested with sugarcane leaves with high carbon content to attain the proper C/N ratio for the production of hydrogen.

After dark fermentation, a large amount of the effluent, also known as hydrogenic effluent, containing high concentrations of volatile fatty acids (VFAs), such as acetic, butyric, and propionic acids, is generated. The chemical oxygen demand (COD) of the hydrogenic effluent has a high COD of approximately 20,000–35,000 mg/L [7, 8], which can cause environmental problems upon discharge. Previous studies have reported the use of hydrogenic effluents to produce bioenergy and biochemicals. For example, a methane yield (MY) of 10.77 mL/g-COD under an optimal hydraulic retention time (HRT) of 9 d was obtained from the hydrogenic effluent of a co-digestion of swine manure and pineapple waste process [9]. The effluent discharged after biohydrogen production from the co-digestion of microalgae with organic wastes, including molasses, Napier grass (Pennisetum purpureum), empty fruit bunches (EFB), palm oil mill effluent (POME), and glycerol waste gave the highest MY of 214–577 mL/g-VS [5]. Hou et al. [10] found that methane production (14.96 kJ/g-VS added) from hydrogenic effluent of food waste supplemented with air-nano-bubble water resulted in a high total energy yield (EY) of 15.31 kJ/g-VS added, which is 43.7 times greater than...
that of hydrogen production (0.35 kJ/g-VS added). These results demonstrate that methane production from hydrogenic effluent significantly improved the total EY. Thus, the two-stage hydrogen and methane production process is a promising process for recovering energy from biomass. In addition to methane, polyhydroxyalkanoates (PHAs) are produced from hydrogenic effluent. Colombo et al. [11] reported that the hydrogenic effluent from the fermentation of second cheese whey (SCW) and concentrated cheese whey permeate (CCWP) was bio-converted to PHAs, resulting in a maximum PHA yield of 0.77 ± 0.14 and 0.72 ± 0.14 mg-COD\textsubscript{PHA}/mg-COD\textsubscript{organic acid-in}, respectively. Zhao et al. [12] found that PHA accumulation of 15.8% of volatile suspended solids (VSS) was achieved from the hydrogenic effluent of original hydrolyzed polyacrylamide-containing wastewater at a C/N ratio of 51. Moreover, PHAs of 40% of the dry cell weight (DCW) were obtained from the effluent obtained after biohydrogen production from distillery waste [13]. These studies indicate the efficacy of VFAs in the production of PHAs. The synthesis of lipids from the hydrogenic effluent has also been studied. Ren et al. [14] found that lipid production from the hydrogenic effluent of stimulated food waste produced the highest lipid production (515.6 mg/L) by Scenedesmus sp. R-16. Mu et al. [15] showed that after the dark fermentation of pretreated duckweed with 1% H\textsubscript{2}SO\textsubscript{4}, the hydrogenic effluent was used to cultivate Chlorella saccharophila FACHB-4, resulting in lipid concentrations and contents of 63.4–270.9 mg/L and 12.0–37.4%, respectively. The production of hydrogen and lipids from starch wastewater by co-culture of oleaginous microalgae with anaerobic sludge was studied by Ren et al. [16]. A maximum total lipid concentration of 0.36 g/L was observed at a starch concentration of 6 g/L, optimal ratio of 30:1, and initial pH of 8. These results indicate the successful utilization of hydrogenic effluent for lipid production by microalgae. However, in these studies of hydrogenic effluent from various types of biomass, only one product, (methane, PHA, or lipid) was obtained. To the best of our knowledge, however, our study is the first to report the use of hydrogenic effluent from Chlorella sp. biomass co-digested with sugarcane leaves to produce multiple products without generating waste (the so-called integrated circular biorefinery concept).

Therefore, the aim of our study was to develop a platform for the utilization of the biomass of Chlorella sp. and sugarcane leaves to produce multiple products (biorefinery concept) including bioenergy, i.e., hydrogen, and methane, and biochemicals, i.e., PHAs, lipid, and soil supplement with the goal to achieve the zero waste generation (circular economy).

**Results and discussion**

**Feedstock compositions and sugarcane leaves pretreatments**

Analysis of Chlorella sp. biomass and its hydrolysate, sugarcane leaf and its hydrolysate, and anaerobic sludge compositions were conducted. The Chlorella sp. biomass consists of (all in % (w/w)): protein 52.8; carbohydrate 29.2; lipid 8.7; and ash 5.0 [17]. The sugarcane leaves are composed of (all in % (w/w)): cellulose 36.69, hemicellulose 17.49, lignin 10.35, and ash 12.62. The main composition found in both Chlorella sp. biomass hydrolysate and sugarcane leaf hydrolysate was carbon, followed by nitrogen and hydrogen (Additional file 1: Table S1). The C/N ratio of the sugarcane leaf hydrolysate was 15.52 and 14.83 times higher than the hydrolysates obtained from Chlorella sp. biomass and anaerobic sludge, respectively. As a result, sugarcane leaf hydrolysate is an excellent choice for balancing the nutrients in the fermentation medium.
Pretreatment conditions of sugarcane leaves by sodium hydroxide (NaOH) and enzyme were optimized to achieve a high sugar yield. The total reducing sugar (TRS) yield increased with increasing NaOH concentration from 0 to 2% (w/v) (Fig. 1). Nevertheless, the TRS yield increased slightly when the NaOH concentration was greater than 2% (w/v) (Fig. 1a). The results suggest that alkaline pretreatment improved the efficiency of cellulose degradation for hydrolysis by enzymes. Generally, alkaline is used to remove lignin from the lignocellulosic biomass structure. The delignification process significantly affects the solubility of hemicellulose [18]. The reaction mechanism is based on the saponification of intermolecular ester bonds, crosslinking lignin and hemicellulose [19]. Saponification allows the breakdown of lignin–carbohydrate complex (LCC) connections and gives the cellulose greater access to the enzyme during hydrolysis [18, 19]. Moreover, pretreatment with NaOH also led to swelling of the cellulose polymeric chain. Consequently, the surface area and accessibility of enzymes to the biomass are increased [20]. The highest TRS yield of 942.8 mg/g-sugarcane leaves was obtained when sugarcane leaves were pretreated with 3% (w/v) NaOH. However, the TRS yield was not significantly different when compared with the TRS yield at NaOH concentrations of 2, 2.5, and 3% (w/v). Thus, a 2% (w/v) NaOH was chosen as the optimal dosage to pretreat the sugarcane leaves. Using low alkaline concentrations would decrease operating costs, chemicals, and waste.

The TRS yield from enzymatic hydrolysis of sugarcane leaves pretreated with the optimum NaOH concentration (2% (w/v)) is depicted in Fig. 1b. Increasing the enzyme loading from 5 to 15 filter paper unit (FPU)/g-sugarcane leaves resulted in a noticeable increase in the TRS yield from 891.2 to 985.5 mg/g-sugarcane leaves. However, the TRS yields did not significantly increase when the enzyme loading was greater than 15 FPU/g-sugarcane leaves (Fig. 1b). The results demonstrate that an enzyme loading higher than 15 FPU/g-sugarcane leaves is not necessary for the hydrolysis reaction. At high enzyme loading (> 15 FPU/g-sugarcane leaves), the adsorption efficiency of the enzyme is reduced by saturation of the enzyme on the cellulose surface [21]. The results indicate that the use of enzyme loading at 15 FPU/g-sugarcane leaves was sufficient to degrade the cellulose structure. Moreover, a low loading of enzymes is preferable over high loading because it lowers the operational costs [2, 21]. Thus, the optimum conditions for alkaline pre-treatment and enzymatic hydrolysis of sugarcane leaves were 2% (w/v) NaOH concentration and 15 FPU/g-sugarcane leaves. Under these conditions, a TRS yield of 985.5 mg/g-sugarcane leaves was achieved, which was 14.1 times higher than that of untreated sugarcane leaves.

### Table 1 Mixture design defining the proportions of Chlorella sp. biomass hydrolysate, sugarcane leaf hydrolysate, anaerobic sludge, and hydrogen production potential results

| Run | Chlorella sp. biomass hydrolysate (g-VS/L) | Sugarcane leaf hydrolysate (g-VS/L) | Anaerobic sludge (g-VS/L) | $P_s$ (mL-H$_2$/L-substrate) | Observed | Predicted |
|-----|------------------------------------------|------------------------------------|--------------------------|-----------------------------|----------|-----------|
| 1   | 6.67                                     | 6.67                               | 6.67                     | 2239                        | 2298     |           |
| 2   | 0.00                                     | 20.00                              | 0.00                     | 2032                        | 2264     |           |
| 3   | 0.00                                     | 10.00                              | 10.00                    | 2454                        | 2478     |           |
| 4   | 16.61                                    | 3.39                               | 0.00                     | 1219                        | 1218     |           |
| 5   | 0.00                                     | 10.00                              | 10.00                    | 2455                        | 2478     |           |
| 6   | 0.00                                     | 20.00                              | 0.00                     | 2471                        | 2264     |           |
| 7   | 3.39                                     | 3.35                               | 13.26                    | 364                          | 216      |           |
| 8   | 3.35                                     | 13.33                              | 3.31                     | 3660                        | 3513     |           |
| 9   | 20.00                                    | 0.00                               | 0.00                     | 1630                        | 1650     |           |
| 10  | 10.00                                    | 0.00                               | 10.00                    | 216                          | 316      |           |
| 11  | 13.27                                    | 3.36                               | 3.37                     | 1964                        | 1818     |           |
| 12  | 0.00                                     | 20.00                              | 0.00                     | 8                             | 21       |           |
| 13  | 0.00                                     | 20.00                              | 0.00                     | 10                           | 21       |           |
| 14  | 10.00                                    | 10.00                              | 0.00                     | 1442                        | 1491     |           |
| 15  | 6.57                                     | 6.79                               | 6.64                     | 2264                        | 2329     |           |
| 16  | 20.00                                    | 0.00                               | 0.00                     | 1646                        | 1650     |           |
| 17  | 10.00                                    | 0.00                               | 10.00                    | 366                          | 316      |           |
| 18  | 6.67                                     | 6.67                               | 6.67                     | 2267                        | 2298     |           |
| 19  | 6.67                                     | 6.67                               | 6.67                     | 2230                        | 2298     |           |

**Biohydrogen production from a co-digestion of Chlorella sp. biomass hydrolysate and sugarcane leaf hydrolysate**

Factors affecting the co-digestion of hydrolysates of Chlorella sp. biomass and sugarcane leaf with anaerobic sludge to produce hydrogen were optimized. D-optimal mixture design was used to design the experiments. The biohydrogen production from the co-digestion of Chlorella sp. biomass hydrolysate ($X_1$), sugarcane leaf hydrolysate ($X_2$), and anaerobic sludge ($X_3$) is presented in Table 1. Regression analysis of the experimental data (Table 1) resulted in the following cubic equation for predicting hydrogen production potential ($P_s$) (Eq. (1)):

$$P_s = 82.49X_1 + 113.18X_2 + 1.05X_3 - 4.66X_1X_2 - 5.19X_1X_3 + 13.36X_2X_3 + 2.80X_1X_2X_3 - 0.37X_1X_2(X_1 - X_2) + 0.83X_1X_3(X_1 - X_3) + 1.85X_2X_3(X_2 - X_3).$$

(1)

The model was significant ($p<0.05$) at the 95% confidence level. The coefficient of determination ($R^2$) of 0.9897 and adjusted $R^2$ of 0.9795 indicate that the model can explain 97–99% of the variability of the response variable (Additional file 1: Table S2). Additionally, the lack of fit of the model is not significant ($p=0.1367$), indicating that the model is valid and properly describes the $P_s$. 

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A three-dimensional (3D) response surface plot based on Eq. (1) was created to determine the interaction effects of the three factors on \( P_s \) (Additional file 1: Fig. S1). The optimum proportions of the feedstock were the hydrolysates of *Chlorella* sp. biomass and sugarcane leaf, and anaerobic sludge of 0.92, 15.27, and 3.82 g-VS/L, respectively. Under these proportions, the maximum \( P_s \) of 3.953 mL-H\(_2\)/L\(_{\text{substrate}}\) was predicted. At these proportions, the C/N ratio was 20.08, which falls within a suitable range of C/N (20-30) for hydrogen production [22]. The confirmation experiment was conducted under the optimal conditions to verify the model. The \( P_s \) of 4.153 mL-H\(_2\)/L\(_{\text{substrate}}\) was observed under optimum conditions, which is only 5.1% different from the predicted \( P_s \). The results suggested that the cubic model was valid for optimizing the feedstock proportions for hydrogen production. At the optimum condition, the observed \( P_s \) (4,153 mL-H\(_2\)/L\(_{\text{substrate}}\)) was 1.5 and 0.8 times higher than mono-digestion of *Chlorella* sp. biomass hydrolysate and sugarcane leaf hydrolysate alone. In addition, it was 414.4 times higher than the mono-digestion of anaerobic sludge. The results demonstrated that the co-digestion process under suitable proportions provided an appropriate C/N ratio and nutrient balance inside the hydrogen fermentation system. A proper C/N ratio of the substrate is essential for the growth and activity of fermentative microorganisms [23]. The excess C/N ratio inhibits the growth and metabolism of microorganisms, resulting in ineffective substrate utilization and hydrogen production. In contrast, a low C/N ratio indicates that the substrate has an excess nitrogen source, which may cause ammonia inhibition and is not suitable for hydrogen production [24].

The results from this study were compared to the literature search which were conducted on a co-digestion of microalgae with various kinds of biomass. We found that the hydrogen yield (HY) obtained under the optimum conditions in this study (207.65 mL-H\(_2\)/g-VS\(_\text{added}\)) was higher than that of the pretreated rice residue co-digested with microalgae (*Chlorella pyrenoidosa*) at a C/N ratio of 17.61 (201.8 mL-H\(_2\)/g-VS) [25], and the macroalgae (*Laminaria digitata*) co-digested with microalgae (*arthropspira platensis*) at a C/N ratio of 26.2 (85.0 mL-H\(_2\)/g-VS) [3]. However, at the same C/N ratio of 20, the HY in this study was 3.75 times greater than HY from the co-digestion of *A. platensis* and *L. digitata* (55.3 mL-H\(_2\)/g-VS) [26]. Results implied that C/N ratios as well as the types of biomass used in the co-digestion process affect the efficiency of hydrogen production.

The synergistic and antagonistic effects of the co-digestion process were investigated by dividing experimental \( P_s \) by calculated \( P_s \) (Table 2). The synergistic effect and the antagonistic effect of mixture proportions are indicated by the ratio higher and lower than 1, respectively, while the ratio is equal to 1 indicates that the substrate works independently from the mixture. The experimental \( P_s \) was obtained from volume of hydrogen produced by each proportion from experimental design while the calculated \( P_s \) was obtained from the VS of each substrate contained in the mixture [6]. An antagonistic effect was found in Runs 2, 4, 7, 10, 12, 14, and 17, while Runs 9 and 16 indicated that the substrate works independently from the mixture (Table 2). In contrast, other experimental runs showed a synergistic effect. At the optimal proportions, an experimental \( P_s \)/calculated \( P_s \) ratio of 2.32 was achieved, indicating a synergistic effect.

| Run | Experimental \( P_s \) (mL-H\(_2\)/L\(_{\text{substrate}}\)) | Calculated \( P_s \) (mL-H\(_2\)/L\(_{\text{substrate}}\)) | Experimental \( P_s \)/calculated \( P_s \) ratio |
|-----|---------------------------------|---------------------------------|---------------------------------|
| 1   | 2239                            | 1299                            | 1.72                            |
| 2   | 2032                            | 2251                            | 0.90                            |
| 3   | 2454                            | 1130                            | 2.17                            |
| 4   | 1219                            | 1742                            | 0.70                            |
| 5   | 2455                            | 1130                            | 2.17                            |
| 6   | 2471                            | 2251                            | 1.10                            |
| 7   | 364                             | 661                             | 0.55                            |
| 8   | 3661                            | 1777                            | 2.06                            |
| 9   | 1630                            | 1638                            | 1.00                            |
| 10  | 216                             | 823                             | 0.26                            |
| 11  | 1964                            | 1466                            | 1.34                            |
| 12  | 9                               | 9                               | 0.93                            |
| 13  | 10                              | 9                               | 1.07                            |
| 14  | 1442                            | 1945                            | 0.74                            |
| 15  | 2264                            | 1305                            | 1.73                            |
| 16  | 1646                            | 1638                            | 1.00                            |
| 17  | 366                            | 823                             | 0.44                            |
| 18  | 2267                            | 1299                            | 1.74                            |
| 19  | 2230                            | 1299                            | 1.72                            |
| Optimum | 3953                         | 1791                            | 2.32                            |

* Experimental \( P_s \)/calculated \( P_s \) ratio < 1; antagonistic effect
* Experimental \( P_s \)/calculated \( P_s \) ratio = 1; the substrate work independently from the mixture
* Experimental \( P_s \)/calculated \( P_s \) ratio > 1; synergistic effect

The results demonstrate that co-digestion at a suitable proportion promotes the interaction between the feedstock and microorganisms, which evidenced by a high \( P_s \). Moreover, synergism in co-digestion may be due to the contribution of enzymes, nutrients, additional trace elements, or any other amendment caused by the co-substrates, resulting in improved biological degradation of substrates and hydrogen production.
Methane production from hydrogenic effluent and energy recovery

The hydrogenic effluent in all experimental runs were further used as a substrate to produce methane in the second stage. Using the hydrogenic effluent under the optimum proportions to produce methane gave the MY of 321.1 mL/g-VS (equivalent to 13.8 kJ/g-VS) (Table 3). The EY under optimum conditions was 6 and 1.2 times, respectively, higher than that of the hydrogen and methane production processes. The results revealed that the two-stage hydrogen and methane production process significantly improved the energy recovery.

The profiles of VFAs and lactic acid of hydrogenic effluent in each experimental run are depicted in Fig. 2. Acetic acid and butyric acid were the primary soluble metabolite products (SMPs) found in all experimental runs, whereas a small amount of propionic acid and lactic acid were found in some experimental runs. Results implied that acetic acid and butyric acid were the primary VFAs being bio-converted into methane by anaerobic mixed cultures in the second stage.

The comparison of HY, MY, and EY of this study with the literature search is shown in Table 4. The HY obtained in this study was relatively high compared to other studies [4, 26, 27]. The differences in HY and MY were due to the types of substrate, inoculum, and fermentation conditions. Fermentation conditions, especially pH, temperature, and nutrients, affected the production yield. Therefore, key factors affecting hydrogen and methane production must be optimized to enhance the production yield.

PHA production

The hydrogenic effluent from the optimum proportions of feedstock that gave the highest $P_8$ was further used for PHA production by Cupriavidus necator. In the treatment with $C. necator$, the biomass concentration rapidly increased and reached a maximum value of 3.80 g/L at 144 h and subsequently decreased over time (Fig. 3a). The PHA concentration rose steadily to the highest value of 2.01 g/L at 168 h and then dropped (Fig. 3b). The decrease in biomass and PHA concentrations could be due to the PHAs stored in bacterial cells being degraded to generate energy for cell maintenance and growth [28]. A maximum PHA content of 60.9% (w/w) and PHA yield of 0.14 g/g-COD$_{consumed}$ were achieved at 168 h in the inoculation treatment (Fig. 3c). The PHA concentration, content, and yield of 0.48 g/L, 27.6% (w/w), and 0.05 g/g-COD$_{consumed}$ were obtained

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**Table 3** Biogas and energy yield (EY) in each run of two-stage hydrogen and methane productions

| Run | Yield (mL/g-VS) | EY (kJ/g-VS) |
|-----|----------------|--------------|
|     | H$_2$ | CH$_4$ | Total | H$_2$ | CH$_4$ | Total |
| 1   | 112.0 | 241.6 | 353.6 | 1.2 | 8.7 | 9.9 |
| 2   | 101.6 | 346.7 | 448.3 | 1.1 | 12.4 | 13.5 |
| 3   | 122.7 | 254.4 | 377.1 | 1.3 | 9.1 | 10.5 |
| 4   | 60.9 | 262.7 | 323.6 | 0.7 | 9.4 | 10.1 |
| 5   | 122.7 | 258.9 | 381.6 | 1.3 | 9.3 | 10.6 |
| 6   | 123.5 | 329.6 | 453.1 | 1.3 | 11.8 | 13.2 |
| 7   | 18.2 | 199.2 | 217.4 | 0.2 | 7.1 | 7.3 |
| 8   | 18.3 | 327.3 | 510.3 | 2.0 | 11.7 | 13.7 |
| 9   | 81.5 | 276.1 | 357.6 | 0.9 | 9.9 | 10.8 |
| 10  | 108 | 225.0 | 353.8 | 0.1 | 8.1 | 8.2 |
| 11  | 98.2 | 290.4 | 388.6 | 1.1 | 10.4 | 11.5 |
| 12  | 0.4 | 115.3 | 115.7 | 0.0 | 4.1 | 4.1 |
| 13  | 0.5 | 127.1 | 127.6 | 0.0 | 4.6 | 4.6 |
| 14  | 72.1 | 312.3 | 384.4 | 0.8 | 11.2 | 12.0 |
| 15  | 113.2 | 259.7 | 372.9 | 1.2 | 9.3 | 10.5 |
| 16  | 82.3 | 271.8 | 354.1 | 0.9 | 9.7 | 10.6 |
| 17  | 18.3 | 194.4 | 212.7 | 0.2 | 7.0 | 7.2 |
| 18  | 113.3 | 252.4 | 365.7 | 1.2 | 9.1 | 10.3 |
| 19  | 111.5 | 276.7 | 388.2 | 1.2 | 9.9 | 11.1 |
| Optimum proportions | 207.7 | 321.1 | 528.8 | 2.3 | 11.5 | 13.8 |
for the control experiment (without the inoculum). The profiles of residual cell concentrations were correlated with the substrate utilization profiles. In the inoculated treatment, residual cell concentration increased rapidly to a maximum of 2.39 g/L at 120 h and then decreased gradually (Fig. 3d). The decrease in residual cell concentration indicated that the feedstock was consumed for PHA production rather than biomass production. The results showed that the VFAs in the hydrogenic effluent could be effectively used to produce PHAs by *C. necator*.

Residual sugar concentration in the treatments with and without *C. necator* was immediately decreased from around 0.3–0.1 g/L within 61 h and gradually decreased to a concentration below 0.1 g/L at the end of fermentation time (Fig. 3e). VFAs were simultaneously consumed with residual sugar, in which total VFAs were completely depleted after 144 and 288 h for inoculum and without inoculum treatment, respectively (Fig. 3f). The results demonstrated that *C. necator* and indigenous microorganisms in the hydrogenic effluent could consume residual sugars and VFAs for biomass and PHA production.

The COD concentration profiles (Fig. 3g) were similar to the total VFAs concentration profiles (Fig. 3f) for both treatments. In the treatment with *C. necator*, the COD concentration continuously decreased during 144 h and remained relatively stable thereafter, whereas the COD concentration in the control treatment decreased steadily
over 288 h (Fig. 3g). The COD was virtually eliminated with removal percentages of approximately 95 and 80% for treatment with and without *C. necator*, respectively. The results demonstrate that the integration production process could convert 97.7% of the COD into hydrogen and PHAs.

The extracted PHA samples were verified using Fourier transform infrared spectrometer (FTIR). The samples showed peaks at wave numbers of 1720.38, 1378.75, 1452.64, and 1274.83 cm\(^{-1}\), corresponding to the C=O bond carbonyl groups, –CH\(_3\), –CH\(_2\), and –CH groups, respectively (Fig. 4a). These peaks correspond to peaks obtained at 1721.00, 1379.36, 1452.73, and 1278.60 cm\(^{-1}\) for standard polyhydroxybutyrate (PHB) (Sigma Aldrich, USA), confirming that the extracted polymer was PHB (Fig. 4b).

In comparison to other literature search, we found that PHA content of this study (60.9% w/w) was equivalent to PHA content produced from the hydrogenic effluent of thermal-hydrolyzed sludge (61.4% w/w) [29] and the
hydrogenic effluent of second cheese whey (62.0% w/w) [11]. Results demonstrated that hydrogenic effluent from *Chlorella* sp. biomass co-digested with sugarcane leaves is a potential feedstock for PHA production.

**Lipid production**

The hydrogenic effluent from the optimal proportions of feedstock was further used to produce lipid by *Acutodesmus* sp. KKU-P2. The strain KKU-P2 gradually grew and achieved a maximum biomass concentration of 1.17 g/L (Fig. 5a), indicating that it could use VFAs in hydrogenic effluent for their growth. The acetic acid concentration decreased significantly over time, whereas butyric acid remained constant until the end of fermentation (Fig. 5b). The results suggested that strain KKU-P2 preferred acetic acid as the carbon source over other acids such as butyric acid. In addition, acetic acid has a two-carbon atom, making it easy to consume by microalgae for biomass and lipid production in this study. This finding was consistent with that of Ren et al. [30], who reported that dark fermentative effluent with acetate as the main SMPs can be used to cultivate microalgal *Scenedesmus* sp. R-16 for lipid production.

The pattern of lipid concentration and content was similar to that of the biomass concentration profile. Lipid concentration steadily increased to the maximum value of 0.20 g/L at 264 h (Fig. 5c) with the highest lipid content of 17.3% (w/w) (Fig. 5d). At this time, the COD was virtually eliminated with a removal percentage of 20%. The fatty acids found in *Acutodesmus* sp. were oleic (C18:1), palmitic (C16:0), and linoleic acids (C18:2). These results were supported by the previous report of Grama et al. [31], who found that oleic, palmitic and linoleic acids were the significant fatty acid fraction found in *Acutodesmus obliquus*. Microalgae growth and lipid production are influenced by many factors, such as substrate concentration, nutrient content, initial substrate to initial biomass concentration ratio (S/X ratio), light, pH, and temperature [14]. Hence, the optimization of these factors can enhance the lipid content and efficiency of COD removal.

**Analysis of composting properties of solid residues at the end of hydrogen fermentation and the remaining acidified slurries from methane fermentation**

The solid residues after the hydrogen fermentation process and the remaining acidified slurries from the methane fermentation process under the optimum proportions of feedstock were analyzed for the composting properties. Results revealed that the chemical properties of the solid residues at the end of hydrogen fermentation passed the compost standard (Table 5). The C/N ratio of 20.22 of the remaining acidified slurries from methane fermentation was slightly higher than the standard of compost (< 20) [32]. Therefore, solid residues
can be directly used as soil supplements without being composted.

The COD flow and market price from a co-digestion of hydrolysate of Chlorella sp. biomass with hydrolysate of sugarcane leaf and anaerobic sludge

The efficiency of organic use and recovery in two-stage hydrogen and methane fermentation (24.7%) was higher than that of hydrogen fermentation alone (68.7%) (Fig. 6a). The results suggest that the two-stage hydrogen and methane production process enhanced the organic recovery, resulting in high energy attained. In addition, the remaining acidified slurries from the methane production process showed properties that could be used as soil supplements.

Figure 6b and c demonstrates other biorefinery schemes in which the hydrogenic effluent was converted to produce PHAs (Fig. 6b) and lipids (Fig. 6c). It was found that 97% of organic waste was utilized to produce hydrogen and PHAs (Fig. 6b), whereas only 65% of the organic waste was converted into hydrogen and lipid (Fig. 6c). Subsequently, the solid residues show the properties of the compost. Therefore, the application of the integrated circular biorefinery concept by producing multi-products was shown to be more viable than a single process, enhanced the COD reduction, and resulted in a complete utilization of the biomass.

The market prices for hydrogen, methane, PHAs, and lipid (linoleic acid) were estimated. The use of hydrolysates of Chlorella sp. biomass and sugarcane leaf, and anaerobic sludge to produce only hydrogen resulted in a gain of approximately 6.09 US$/kg; in contrast, the production of only methane resulted in a market price gain of 0.24 US$/kg [33] (Additional file 1: Fig. S2). Hence, the integration of two-stage hydrogen and methane results in a market price gain of 6.33 US$/kg. VFAs with a market price of 1.0 US$/kg can be conveniently used as a precursor for PHA and lipid (linoleic acid) synthesis. The market products of PHA and linoleic acid synthesis can have a value of up to 6.09 [33] and 4.0 [34] US$/kg, respectively. Therefore, the integration of hydrogen production with PHAs or lipid synthesis increases the net market price of 12.18 and 10.09 US$/kg, respectively. To commercialize these processes, it is essential to recognize the bottlenecks of processes, such as operational costs, extraction performance, and harvest.

Conclusions

Biohydrogen production followed by methane, PHA and lipid production is a successful integrated circular biorefinery platform to efficiently utilize the hydrolysates of Chlorella sp. biomass and sugarcane leaf. The potential use of the solid residues at the end of hydrogen fermentation and the remaining acidified slurries from methane production as soil supplements demonstrates the zero waste concept. The COD flow clearly indicated that integrated circular biorefinery concept to produce value-added multi-products is more viable than a single process. The approach we have demonstrated in this study provides a foundation for the maximum use of feedstock, resulting in zero waste.
Table 5 Comparison of standard compost with chemical properties of solid residues after the hydrogen fermentation process and the remaining acidified slurries from the methane fermentation process

| Characteristic                  | Criteria | Solid residues from hydrogen production | Remaining acidified slurries from methane production |
|--------------------------------|----------|-----------------------------------------|----------------------------------------------------|
| Electrical conductivity (dS/m) | ≤ 6      | 3.95                                    | 5.32                                               |
| Organic matter (%w/w)          | ≥ 30     | 88.92                                   | 89.27                                              |
| Organic carbon (%w/w)          | –        | 51.57                                   | 51.78                                              |
| C/N ratio                      | ≤ 20     | 12.58                                   | 20.22                                              |
| Germination index (%w/w)       | ≥ 80     | 98                                      | 87                                                 |
| Total N (%w/w)                 | ≥ 1.0    | 4.10                                    | 2.56                                               |
| Total P₂O₅ (%w/w)              | ≥ 0.5    | 2.41                                    | 1.89                                               |
| Total K₂O (%w/w)               | ≥ 0.5    | 1.94                                    | 1.28                                               |

Fig. 6 The COD flow and mass balance analysis of organic matter (COD) throughout the different biorefinery schemes. 

a Two-stage hydrogen and methane production; 
b hydrogen and PHA synthesis; c hydrogen and lipid synthesis
Materials and methods

Inoculum preparation

Anaerobic microorganisms contained in anaerobic sludge were used as the inoculum for hydrogen production. They were kindly provided by Kaensiri Starch Co., Ltd. Phra Yuen, Khon Kaen, Thailand. The sludge was heat-treated at 105 °C for 3 h in a hot air oven to inactivate methane-producing bacteria. The sludge was then activated and enriched in a modified basic anaerobic (BA) medium [35] with 5 g/L of glucose as a carbon source in a glass bottle of 400 mL working volume. The initial pH was adjusted to 6.0, using either 5 M NaOH or 5 M HCl. Rubber stoppers and aluminum caps were used to seal the bottles. Anaerobic conditions inside the glass bottle were created by purging with nitrogen gas for 10 min. The culture was incubated at room temperature (30±2 °C) and 150 rpm for 24 h on an orbital shaker. The culture was then transferred to a new BA medium (subculture) and incubated for 24 h. After the enrichment culture was complete, the culture was centrifuged at 5974 g for 10 min to harvest the sludge.

For methane production, anaerobic microorganisms contained in anaerobic sludge were used as the inoculum without heat treatment and were cultivated in the medium, as previously described. The initial pH of the BA was adjusted to 7.0, using 5 M NaOH and 5 M HCl. The culture was incubated at 20 °C in a shaking water bath at 150 rpm for 24 h. The cells were then harvested by centrifugation (3000 g 4 °C for 15 min to isolate the cells for use in lipid production.

C. necator (former name: Alcaligenes eutrophus TISTR 1335), purchased from the Thailand Institute of Scientific and Technological Research (TISTR), Thailand, was used as the inoculum for PHA production. It was grown in nutrient broth (NB) using 20 g/L glucose as the carbon source. The culture was incubated at 30 °C and 200 rpm for 18 h. The cells were harvested by centrifugation at 3,500 g 4 °C for 15 min, and re-suspended in the hydrogenic effluent before use.

Acetobacterium sp. KKU-P2 (GenBank Accession No. MW555785), kindly provided by Dr. Pensri Plangklang, was used as an inoculum for lipid production. It was isolated from a freshwater fish farming area, Nakhon Ratchasima, Thailand, using a modified Bold’s basal medium [36] with 4.4 mM NaNO₃ as a nitrogen source and 30% CO₂ in the air as a carbon source. The inoculum was cultivated in Bold’s basal medium [36] with 10 g/L glucose as the carbon source. The culture was incubated in an orbital incubator shaker at 30 °C and 150 rpm under a light intensity of 3,000 lx for 4 days. The cells were then centrifuged at 3500 g 4 °C for 15 min to harvest cells for use in lipid production.

Chlorella sp. biomass was purchased as a dry powder from Yantai Hearol Biotechnology Co., Ltd. Chengmai, Hainan, China. It was stored at −20 °C in zipper storage bags until use. Before use, the samples were pretreated using an enzymatic pretreatment method. Briefly, 2.6 g of Chlorella sp. biomass was added to 200 mL of distilled water. Mixed enzymes, including cellulase (Cellic® CTec2), glucoamylase (Dextrozyme® GA), and alpha-amylase (Termamyl® SC) from Novozyme, Denmark, were applied to the glass bottle in the appropriate proportions, and the conditions were set according to Giang et al. [37].

Sugarcane leaves were collected from a local sugarcane field in Khon Kaen, Thailand. The sugarcane leaves were milled and sieved through a 0.5-mm screen. Before use, the milled sugarcane leaves was pretreated with alkaline pretreatment followed by enzymatic hydrolysis. Sugarcane leaves (10 g) were pretreated with 100 mL of NaOH (1:10 (w/v)) at various NaOH concentrations (0.5, 1, 1.5, 2, 2.5, and 3% (w/v)). Pretreatment was performed in an autoclave at 121 °C for 60 min. After alkali pretreatment, the solid fraction was filtered through a muslin cloth, rinsed with tap water until pH 7–8, and then dried at 80 °C for 24 h in a hot air oven. Subsequently, 2.5 g of pretreated sugarcane leaves were hydrolyzed with Cellic® CTec2 (Novozyme) at a 35 FPU/g-sugarcane leaves in 50 mL of 0.05 M sodium citrate buffer (pH 5.0) [2] and incubated in a shaking water bath at 50 °C and 150 rpm for 72 h. The TRS concentrations of sugarcane leaf hydrolysate were analyzed using the 3,5-dinitrosalicylic acid (DNS) method. The conditions that gave the highest TRS concentration were further used to optimize the enzyme loadings of 5, 15, 25, 35, 45, 55, and 65 FPU/g-sugarcane leaves. The enzymatic hydrolysis process was conducted as described previously. Subsequently, TRS concentrations in sugarcane leaf hydrolysates were analyzed.

Statistical design for biohydrogen production

A D-optimal mixture design was applied to determine the optimum proportions of feedstock for hydrogen production. Based on the total initial substrate concentration of 20 g-VS/L, the proportions of Chlorella sp. biomass hydrolysate (X₁), sugarcane leaf hydrolysate (X₂), and anaerobic sludge (X₃) were varied according to the design (Table 2) using Design-Expert software (Demo Version 7.0, Stat-Ease Inc., Minneapolis, MN, USA). The hydrogen production potential (Pₚ) was used as a response (Table 2). The cubic model
describes the relationship between the independent variables, and the response variable is shown in Eq. (2):

\[
Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k + \sum \delta_{ij} X_i X_j (X_i - X_j),
\]

(2)

where \(Y\) is the predicted response, \(X_i, X_j, \) and \(X_k\) are the independent variables, \(\beta_0\) is a constant, \(\beta_i\) is the coefficient of individual factors, \(\beta_{ij}\) and \(\delta_{ij}\) are the coefficients of two interacting factors, and \(\beta_{ijk}\) is the coefficient of the three interacting factors.

Two-stage hydrogen and methane production

Batch hydrogen fermentation was carried out in 120-mL serum bottles with a working volume of 70 mL. The feedstock was then added to the bottles, as shown in Table 1. The bottles were then filled with a modified BA medium [35] and the working volume was adjusted to 70 mL using distilled water. The initial pH was adjusted to 6.0, using either 5 M NaOH or 5 M HCl. The bottles were sealed with rubber stoppers and aluminum caps. Subsequently, nitrogen gas was flushed into the headspace for 10 min to create anaerobic conditions. All bottles were incubated on an orbital shaker at 30 °C and 150 rpm. All experimental runs were performed in triplicate. During fermentation, the biogas volume was measured using the wetted glass syringe method [38]. The fermentation broth was collected to analyze the concentration of VFAs at the end of fermentation.

Hydrogenic effluent in each experimental run were further used as the substrate for methane production in the second stage. Methane batch fermentation was conducted by adding 30 mL of hydrogenic effluent and 10 g-VS/L of anaerobic sludge into a 60-mL serum bottle. The pH was adjusted to 7.0, using 5 M NaOH. All bottles were sealed and flushed with nitrogen gas again to create anaerobic conditions and incubated under the same conditions as mentioned above. The biogas volume was measured as previously described.

PHAs and lipid production

Hydrogenic effluent from the optimum proportions of feedstock that gave the highest hydrogen production was used as the substrate for PHA and lipid production. The effluent was centrifuged at 5,974 g for 10 min to remove all suspended solids, and only the supernatants were collected. PHA fermentation was performed in 500-mL Erlenmeyer flasks containing 200 mL of hydrogenic effluent (supernatants), 30% (v/v) of \(C. \) necator (initial cell concentration \(10^7\) CFU/mL), and an initial pH of 7.0. The flasks were incubated in an orbital incubator shaker at 30 °C and 200 rpm for 12 days. Samples were taken during fermentation to analyze the concentrations of sugar residues, VFAs, biomass, PHAs, and COD. The control experiment was performed in the same manner but without inoculums.

Lipid production was performed in 500-mL Erlenmeyer flasks containing 200 mL of sterile supernatant with 0.75 g/L of NaNO₃ as a nitrogen source and 0.2 g-DCW/L of Acutodesmus sp. KU-KU-P2 as inoculum. The initial pH was adjusted to 7 using a 5 M NaOH solution. The flasks were incubated at 30 °C and 150 rpm under a light intensity of 3000 lx for 14 days. The control test was conducted in the same manner without KU-KU-P2. During fermentation, the fermentation broth was collected to analyze the biomass and lipid concentrations, lipid content, and VFAs concentration.

The potential use of solid residue and the remaining acidified slurries from two-stage hydrogen and methane production process as soil supplements

The solid residues after hydrogen production and the remaining acidified slurries or mixtures from the methane production process under the optimum proportions were collected to analyze the composting properties, including electrical conductivity, organic matter, organic carbon, C/N ratio, germination index, total nitrogen (N), total phosphorus pentoxide (P₂O₅), and total potassium oxide (K₂O). The analytical values were compared with standard compost values to assess their compost properties.

Analytical methods

The CHNS/O content of hydrolysates of Chlorella sp. biomass and sugarcane leaf and anaerobic sludge was analyzed using a CHNS/O analyzer (Flash 2000, Thermo-Scientific, Italy) at the Scientific Equipment Center, Prince of Songkla University, Thailand. Total solids (TS), VS, moisture, and ash content were determined using standard methods [39]. The pH and COD were measured using a pH meter (pH-500, Queen, USA) and a SpectroQuant® COD cell test kit (Merck, Germany), respectively. Cellulose, hemicellulose, and lignin contents were analyzed following the method described by Goering and Van Soest [40]. The total sugar concentration was measured using the phenol–sulfuric acid method [41], and the TRS concentration was determined using the DNS method [42] with glucose as a standard. The TRS yield of sugarcane leaf hydrolysate was calculated by subtracting the TRS concentrations of the hydrolysate by the TRS concentration of the enzyme. The lipid content of microalgae was determined using the colorimetric sulfo-phospho-vanillin (SPV) method [43]. The electrical conductivity was determined using a conductivity/DO meter (CyberScan
The online version contains supplementary material available at https://doi.org/10.1186/s13068-021-02041-6.

Additional file 2: Table S2. ANOVA for the cubic model regression representing hydrogen production potential in mixture design.

Additional file 3: Figure S1. The mixture design plots for hydrogen production potential from the co-digestion process. (a) 3D surface; (b) contour.

Additional file 4: Figure S2. The hydrogen, methane, PHAs, and lipid (linoleic acid) production and the market prices of various products.

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Authors’ contributions
NS was involved in designing the study, performed the experiments under the supervision of AR, and wrote the first draft of the manuscript. SS assisted with data analysis and revised the manuscript. SK analyzed the data and provided advice on soil supplements. AR conceived of the project, assisted in the design of experiments, supervised the research, and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors agree to the publication of this manuscript.

Competing interests
The authors declare that they have no competing interests.

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