

Enhancing energy recovery via two stage co-fermentation of hydrothermal liquefaction aqueous phase and crude glycerol

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ABSTRACT

Hydrothermal liquefaction (HTL) is a promising method to convert wet biomass into biocrude oil which can further be upgraded into transportation fuel. Approximately 20–40% of the total energy still remains in the aqueous phase after the HTL process. While conventional anaerobic digestion has demonstrated a limited conversion efficiency, two stage co-fermentation with crude glycerol was developed in this study to process HTL aqueous phase (HTL-AP) into hydrogen and methane, aiming to enhance biogas generation and energy recovery. Compared with single stage operation, two stage HTL-AP fermentation improved the biogas production by 25.5%. Subsequently, the addition of co-substrate crude glycerol helped relieve the acidic stress, adjusted the nutrient supply, and diluted the toxic concentration of chemicals in HTL-AP within the reactors. The biogas production was further enhanced by 1.85 times from single stage when the HTL-AP to crude glycerol ratio was 1:1. The initial pH value of the two stage operation was also controlled to optimize the metabolic pathways during the first stage of hydrogen production and to provide desirable intermediates for methanogenesis. Results showed that an initial pH of 5.5 resulted in the highest hydrogen production in this study. Accompanied with the enhanced biogas yield, the organic conversion, energy generation, and energy recovery from two stage co-fermentation were improved by 48.6%, 84.9%, and 40.1% compared to single stage fermentation, respectively. The enhanced biogas production, especially the hydrogen generation, provided a promising direction for wet biomass conversion. Specifically, downstream two stage treatment of HTL-AP could be integrated with upstream HTL by utilizing the produced hydrogen for upgrading biocrude oil via hydrocracking, and the methane could be used as a heating source for the HTL process.

1. Introduction

Hydrothermal liquefaction (HTL) is a thermochemical process that converts wet biomass into biocrude oil under a temperature of 250–370 °C and a pressure of 4–25 MPa [1,2]. It is particularly suitable for wet substrates because it does not require a drying pre-treatment, and the high temperature helps to kill the pathogens and toxins in the substrate, reducing their negative effects on the environment [3,4]. The oil yield, higher heating value, and the energy recovery rate have previously achieved values of 80 wt%, 30 MJ/kg, and 50% through HTL, respectively [4,5]. The biocrude oil can be further upgraded by hydrocracking, distillation, and esterification into biodiesel which shares similar chemical properties with petroleum diesel [6]. Although the

prospects of HTL are promising, valorization of the HTL aqueous phase (HTL-AP), a by-product which contains 35–40% of the feedstock carbon and 65–70% of the feedstock nitrogen, clouds the commercial application of this thermochemical technology [7,8]. HTL-AP is mainly composed of organic acids, aldehydes/ketones, and nitrogen-containing compounds [9], and it exhibits a toxic nature that can be attributed to the abundant nitrogen-containing and aromatic compounds. To recover the nutrients and energy, anaerobic digestion has been performed to chemically convert HTL-AP into a renewable biogas [10,11]. The biogas yield from anaerobic digestion could range from tens to over 300 mL methane/g COD, depending on the feedstock and reaction conditions [12]. However, the handling capacity of conventional anaerobic digestion is limited by a low organic degradation efficiency of 33–64% and a

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high dilution rate of 5–1000 to reduce the toxicity of HTL-AP [13]. With these restrictions, several strategies have been explored to optimize the process. For example, ozone was used as a pretreatment that greatly enhanced the organic conversion and methane generation [13]. Adding adsorbents such as activated carbon and natural zeolite to adsorb the inhibitory substances in HTL-AP also showed great potential to improve digestion performance [11,14,15]. These techniques greatly promoted the process efficiency, but they also challenged the reactor design and economic feasibility of anaerobic digestion. From a technical and economic aspect, another strategy named two stage fermentation has been proposed that could greatly improve anaerobic digestion efficiency.

Compared with single stage operation, two stage fermentation divided the process into two distinct stages. In the first stage, a hydrogen-rich biogas was produced along with intermediate organic acids and alcohols. In the second stage, these organic intermediates were subsequently converted into methane. Two stage fermentation could handle a higher organic loading rate, which may lead to process failure during traditional single stage reactions. Moreover, stage separation could reduce the hydraulic retention time while concomitantly improving the energy conversion and promoting biogas generation, especially hydrogen [16–20]. The enhancement of two stage operation could be attributed to the interconnections between these two stages. To be specific, the enhanced hydrogen production of the first stage provided a suitable environment and reaction intermediates, such as VFAs and ethanol, for acetogenesis and methanogenesis. Hydrogen production served as a detoxification step that reduced the toxin concentration in HTL-AP and mitigated their negative effects on microbial communities [18,21]. For example, more than 90% of the furan derivatives could be degraded with an initial concentration less than 1 g/L while simultaneously increasing the hydrogen productivity [21], and this process helped to alleviate the toxic inhibition on acetogens and methanogens. Moreover, the decreased hydrogen partial pressure with hydrogen generation in the first stage favored acetogenesis and acetoclastic methanogenesis in the second stage [22]. Therefore, two stage operation provided a more stable system for a considerably higher biogas production. The produced biohydrogen and biomethane could

serve as value-added products or energy alternatives, and these products could reduce environmental pollution and greenhouse gas emissions caused by the massive use of conventional fossil fuels [23,24].

Addition of a co-substrate has also been proposed for two stage fermentation to further increase organic conversion (Table 1). Previous studies on two stage co-fermentation of hydrothermal carbonation aqueous phase and corn stover reported that the methane production increased by 10.69% in comparison to single stage fermentation of the aqueous phase [25], indicating that this strategy could also be a promising solution to deal with HTL-AP for energy restoration. The fact that HTL-AP containing glycerol was an ideal anaerobic digestion feedstock for biogas production [7,26] confirmed the potential of glycerol as a co-substrate. Crude glycerol is an organic waste from the generation of biodiesel. In total, around 100 g of crude glycerol can be produced per kilogram of biodiesel [27]. As an easily degradable carbon source, digestion of crude glycerol has attracted a great amount of attention [28,29]. Hydrogen production is particularly favored through glycerol conversion, and many of the anaerobes performed better with crude glycerol because of the impurities [28]. The capacity of crude glycerol to be co-fermented with other substrates has been demonstrated, where the co-fermentation of crude glycerol and decanter cake helped improve the production of hydrogen and methane by 75% and 153%, respectively [30]. In terms of the co-fermentation of HTL-AP and crude glycerol, on the one hand, addition of crude glycerol could bring a carbon source to the feedstock and balance the great amount of nitrogen supplied by HTL-AP. On the other hand, the alkaline nature of glycerol could neutralize the low pH and ease acidic stress caused by HTL-AP. However, no previous study has investigated the detailed effects of crude glycerol on HTL-AP digestion, and the related co-fermentation mechanisms are still unknown.

Herein, this study is the first to propose two stage co-fermentation of HTL-AP and crude glycerol for hydrogen and methane production. In order to have a deeper and more comprehensive understanding of the co-fermentation process, this study evaluates the influence of two stage operation on the hydrogen production, methane production, enhanced organic conversion, and energy recovery during this process.

Table 1

Summary of biohydrogen and biomethane generation from two stage co-fermentation of waste organics.

Substrates	pH	Mix ratio	Hydrogen yield	Methane yield	Energy generation	Reference
<i>Chlorella</i> biomass & molasses,	NA	1: 0.02–0.06 (w/w)	17–75 L/kg-VS	214–577 L/kg-VS	NA	[35]
palm oil mill effluent, glycerol						
Crude glycerol & decanter cake	7.0	1.33–2.66: 1 (w/w)	23 L/kg-TS (1.5% glycerol)	44 L/kg-TS (0.75% glycerol)	0.056 KWh/kg-TS	[36]
Glycerol & sewage sludge	Initial 6.8	2% v/v glycerol	NA	483 L/kg-VS	NA	[37]
Hydrothermal carbonation	7.0	Optimal mass ratio	NA	280.7 L/kg-VS	NA	[25]
wastewater & corn stover		2:1				
Glycerol & organic fraction of	1st stage: 4.5–5.5;	1% v/v glycerol	26 L/kg-VS	367 L/kg-VS	NA	[30]
municipal solid waste	2nd stage: 6.8–7.0					
Glycerol & olive mill +	1st stage: 4.1–5.6;	1% v/v glycerol	15 L/kg-VS	190 L/kg-VS	NA	[30]
slaughterhouse wastewater	2nd stage: 6.9–7.6					
(1:4)						
Skim latex serum & palm oil mill	1st stage: 5.9–6.1	55: 45 (g-VS)	84.5 L/kg-VS	311.2 L/kg-VS	12.22 kJ/g-VS	[38]
effluent						
Cassava starch wastewater &	Optimal 6.8	Optimal COD/total	16.9 L/kg-COD	NA	NA	[39]
buffalo dung		nitrogen ratio 42.36				
Garden waste & food waste	7.0–7.2 (2nd stage)	9:1 (w/w)	46 ± 1 L/kg-VS	682 ± 14 L/kg-VS	24.9 MJ/kg-VS	[40]
Grass/silage & cow dung	1st stage: 6.0; 2nd	3:1 (g-VS)	27.71 L/kg-VS	370.99 L/kg-VS	480.27/204.70 MJ/g-	[41]
	stage: 7.5				VS	
Food waste & brown water	1st stage: 5.0–5.5;	7:3 (w/w)	99.8 L/kg-VS	728 L/kg-VS	26.5 kJ/g-VS	[42]
	2nd stage: 7.0–7.5					
Macro- & micro algal biomass	1st stage: 6.0; 2nd	C/N ratio is 20:1	97 L/kg-VS	295.9 L/kg-VS (biomethane	Max energy	[43]
	stage: 8.0			potential)	conversion efficiency	
					70.9%	
Waste activated sludge & food	6.9–7.7	1:3 (v/v)	NA	316 L/kg COD (thermophilic);	NA	[44]
wastewater				268 L/kg COD (mesophilic)		
Food waste & sewage sludge	1st stage: 6.0; 2nd	3:1 (g-VS)	174.6 L/kg-VS	264.1 L/kg-VS	11.3 kJ/g-VS	[45]
	stage: 8.0					

VS: volatile solid, TS: total solid, NA: not available.

Specifically, since pH and the substrate composition greatly affect the metabolic activities of specific microbes and the organic conversions [31–34], the effects of initial pH values, the optimal mix ratio of HTL-AP and crude glycerol, and their synergistic enhancements on fermentation were investigated, aiming to provide a reference for higher biogas yields and energy conversions during large scale continuous applications.

2. Materials and methods

2.1. Sample preparation

The HTL-AP was collected after a pilot scale HTL reaction derived from swine manure. The HTL experiment was conducted at a temperature of 270 ± 10 °C with a retention time of 1 h. The HTL-AP was kept at room temperature and filtered by a $0.45 \mu\text{m}$ filter before use.

The inoculum was collected at Urbana & Champaign Sanitary District (Urbana, Illinois, USA) after anaerobic digestion of a synthetic wastewater (1 g COD/L) [19]. The total solids and volatile solids of the inoculum were $2.68 \pm 0.07\%$ and $64.19 \pm 0.07\%$, respectively.

Crude glycerol was collected from a biodiesel plant (Illinois, USA), in which crude glycerol was produced as the by-product from biodiesel from transesterification of campus waste cooking oil. The characteristics of HTL-AP and crude glycerol are summarized in Table 2.

2.2. Experimental design

Seventeen groups of experiments were conducted as shown in Table 3. Effects of initial pH values (4.5, 5.5, and 6.5) and substrate mix ratios (HTL-AP to crude glycerol = 1:0, 3:1, 1:1, 1:3, 0:1, on a COD basis) were investigated in this study. In addition, single stage anaerobic digestion of HTL-AP and crude glycerol as sole substrates were also monitored as control groups.

Serum bottles with an effective working volume of 160 mL were used for digestion. The inoculum for hydrogen production was pre-heated at 100 °C for 2 h. HTL-AP and crude glycerol were mixed in 5 different ratios (on a COD basis) with a total substrate concentration of 10 g COD/L. The initial pH values were only adjusted before fermentation to 4.5, 5.5, and 6.5 with NaHCO_3 addition for each group (Table 3). Reactions were operated at 37 °C using a water jacket for 6 days during the first stage. Then, another 30 mL fresh inoculum was added for methane production. The second stage fermentation was also performed at 37 °C for 29 days. Gas volume was measured daily with a glass syringe, and the gas content was measured daily by gas chromatography.

2.3. Analytical methods

A modified Gompertz model was used to fit the methane production curve [46]:

$$M = P_{\max} \cdot \exp\left\{-\exp\left[\left(R_{\max} \cdot \frac{e}{P_{\max}}\right) \cdot (\lambda - t) + 1\right]\right\}$$

Table 2
The characteristics of HTL-AP and crude glycerol.

	HTL-AP	Crude glycerol
pH	4.53 ± 0.03	9.25 ± 0.02
COD	27300 ± 624 mg/L	1.60 ± 0.02 g/g
Total N	1603 ± 524 mg/L	UD
$\text{NH}_3\text{-N}$	80 ± 3.9 mg/L	UD
Formic acid	UD	UD
Acetic acid	5468 ± 41 mg/L	UD
Propionic acid	988 ± 17 mg/L	UD
Butyric acid	1680 ± 2 mg/L	UD
Valeric acid	336 ± 9 mg/L	UD
Glycerol	UD	0.711 ± 0.013 g/g

UD = under detection limit.

where M is the cumulative methane yield at fermentation time t (mL/g COD), $P_{\max}$ is the maximum methane yield potential (mL/g COD), $R_{\max}$ is the maximum methane production rate (mL/(g•d) COD), λ is the lag phase (d), and e is 2.71828. OriginPro was used to fit the model and results.

Chemical oxygen demand (COD) was measured by the Hach method using a Hach spectrophotometer (Model DR3900). High performance liquid chromatography (HPLC) (Shimadzu Scientific Instruments, Japan) was performed to monitor the concentrations of volatile fatty acids (VFAs) including acetic acid, propionic acid, butyric acid, valeric acid, as well as glycerol. An Aminex HPX-87H column (Bio-Rad, USA) was used and kept at 40 °C while the mobile phase was 5 mM H_2SO_4 .

Total energy generation (E) was calculated as [47]

$$E = P_{\text{H}_2} \cdot \rho_{\text{H}_2} \cdot HHV_{\text{H}_2} + P_{\text{CH}_4} \cdot \rho_{\text{CH}_4} \cdot HHV_{\text{CH}_4}$$

where P_{H_2} and P_{CH_4} are the yields of hydrogen and methane tested in this study, respectively; ρ_{H_2} and ρ_{CH_4} are the densities of hydrogen and methane at 0 °C and 1 bar, which are 0.090 g/L and 0.716 g/L, respectively; HHV_{H_2} and HHV_{CH_4} are the higher heating values of hydrogen and methane, which are 141.7 MJ/kg and 55.5 MJ/kg, respectively [48].

Energy recovery was calculated based on the methane yield which is the ratio of the actual methane yield to the theoretical methane yield (350 mL/g COD).

The correlation between the operation parameters (stage operation, initial pH, and mix ratio) and biogas production (hydrogen yield, methane yield, lag phase, and methane production rate) was visualized using principal component analysis (PCA) and a correlation plot. OriginPro was used to present a 3D PCA plot, and R function rquery.cormat was used to provide the correlation plot.

3. Results and discussion

3.1. Promoted biohydrogen generation in two stage co-fermentation

Fig. 1 shows the hydrogen production from the first stage of co-fermentation, which was highly dependent on the initial pH value in the reactors. The first stage of co-fermentation functioned to produce hydrogen and provide the desirable intermediates for the second stage. The best promotion of the hydrogen yield from the co-fermentation of HTL-AP and crude glycerol was observed with an initial pH of 5.5 (Fig. 1 (b)). For reactions with a pH of 6.5 and a pH of 4.5, although LE (Fig. 1 (a)) showed a promising hydrogen yield with glycerol as the sole substrate, the other hydrogen productions were lower than 1.99 and 0.19 mL/g COD with HTL-AP. These results were consistent with previous studies which demonstrated that the suitable pH for hydrogen production was 5.0–6.0 and the optimal pH was 5.5–5.7. The growth of hydrogen producing bacteria would be inhibited while the hydrogen metabolic pathway was affected if the pH was lower than 5.0 [38,49,50]. Moreover, most of the ATP was used to maintain the pH balance inside microbial cells for survival instead of hydrogen production under inadequate pH conditions [39,51].

The performance of hydrogen generation under different initial pH conditions could further be explained with intermediates production in Fig. 2. Specifically, the macro organics were degraded and the concentration of the accumulated VFAs were higher at a pH of 6.5 than at pH 4.5 and 5.5. But hydrogen generation was inhibited at this pH condition. This might be due to the homoacetogenic process under this high pH condition that converts hydrogen into acetic acid, and the homoacetogenic process was further strengthened with an increased biomass density due to crude glycerol addition [52,53]. Therefore, the rapid hydrogen consumption overwhelmed generation at a pH of 6.5, leading to the low hydrogen yield. As for the pH of 4.5, VFAs did not accumulate in the reactors with HTL-AP (LA-LD in Fig. 2), which demonstrated that the low pH inhibited the activity of microbial communities and the

Table 3
Experimental design.

Group	Two Stage															Single Stage	
	LA	LB	LC	LD	LE	MA	MB	MC	MD	ME	HA	HB	HC	HD	HE	G	P
pH	4.5	4.5	4.5	4.5	4.5	5.5	5.5	5.5	5.5	5.5	6.5	6.5	6.5	6.5	6.5	7.9	6.1
R	1:0	3:1	1:1	1:3	0:1	1:0	3:1	1:1	1:3	0:1	1:0	3:1	1:1	1:3	0:1	0:1	1:0

L, M, H indicate that the initial pH values of the reactors are 4.5, 5.5, and 6.5; A, B, C, D, E indicate that the R values of the reactors are 1:0, 3:1, 1:1, 1:3, and 0:1; R is the ratio of HTL-AP to crude glycerol; 1:0 means HTL-AP is the sole substrate; 0:1 means crude glycerol is the sole substrate.

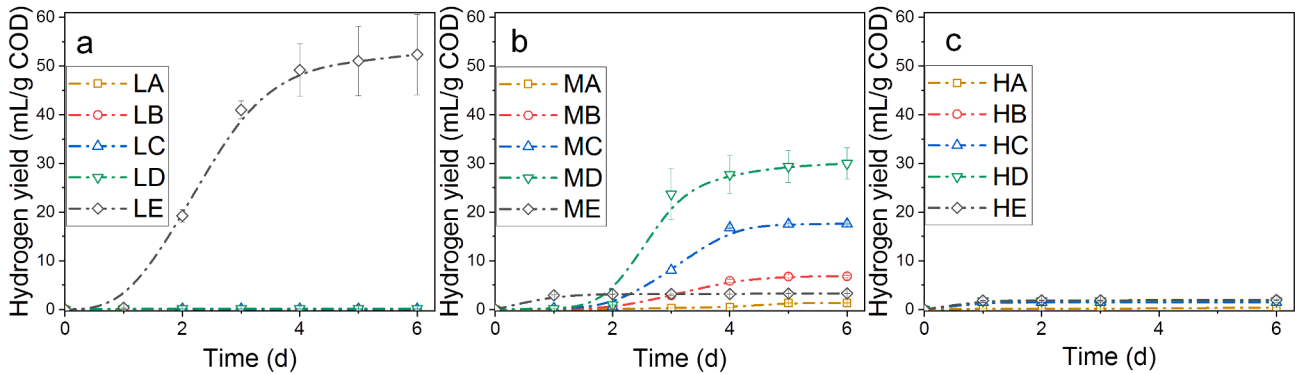


Fig. 1. Hydrogen production from co-fermentation at a pH of 4.5 (a), at a pH of 5.5 (b), and at a pH of 6.5 (c).

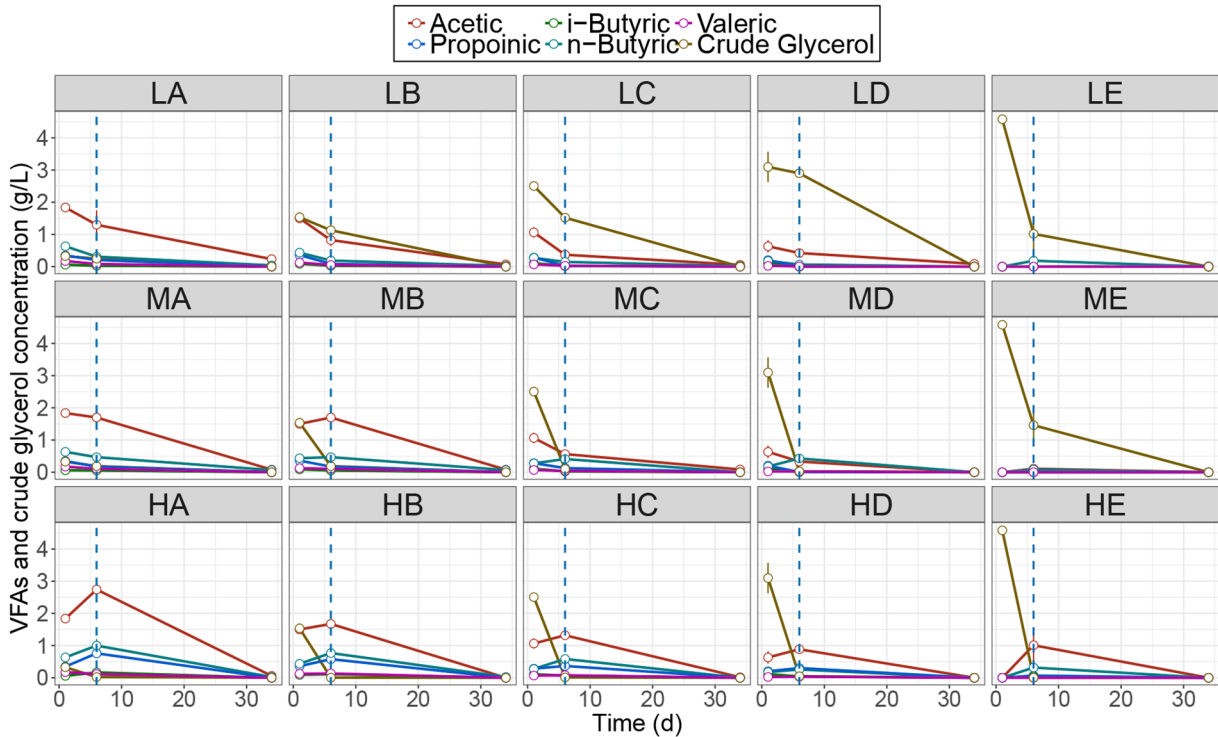


Fig. 2. VFAs and crude glycerol concentrations during two stage co-fermentation at a pH of 4.5 (first line), a pH of 5.5 (second line), and a pH of 6.5 (third line). The blue dotted line separates the first stage from the second stage.

typical metabolic pathways. Meanwhile, the low pH favored the existence of undissociated acids, then solvents were produced instead of hydrogen once the undissociated acid concentration reached a critical value [31,54]. It's also worth noting that the initial VFAs concentration was relatively high in HTL-AP, then the acidic inhibition on hydrogen production would be stronger in groups with higher HTL-AP concentration (Fig. 2). On the contrary, for LE with crude glycerol as the sole substrate, the concentration of acids was lower while glycerol

predominated that favored hydrogen yield within the reactors. Notably, an accumulation of butyric acid was observed in LE, and similar trends were observed for a pH of 5.5, indicating that butyrate type fermentation could be the key mechanisms for hydrogen production.

In addition to the initial pH adjustment, co-fermentation of crude glycerol benefited biohydrogen production (Fig. 1). Crude glycerol is a great carbon source that can be easily degraded during fermentation. The end products of this process are hydrogen, carbon dioxide, VFAs,

and alcohols such as 1,3-propanediol [55]. Results demonstrated that the hydrogen yields were improved 5.0 and 12.9 times higher with a HTL-AP to crude glycerol ratio of 3:1 and 1:1 compared to the two stage fermentation of HTL-AP itself. And the highest hydrogen yield of 30.0 mL/g COD can be obtained as the content of crude glycerol in the substrate increased to 75% at the pH of 5.5. The improvement can be explained by the enhanced system capacity with co-substrate addition. Crude glycerol addition may induce an increased microbial diversity, leading to an enhanced hydrolysis rate which benefits the overall digestion efficiency [56]. A consistent promotion was reported by Jehlee et al. (2019) that co-fermentation of glycerol increased the cell wall digestibility and microbial diversity [35]. The enhanced hydrogen generation with the addition of crude glycerol appeared at all pH conditions. One possible reason for this positive correlation was the buffering effect of crude glycerol during co-fermentation. The metabolism of crude glycerol produced 1,3-propanediol, and a higher glycerol content in the substrate resulted in a higher 1,3-propanediol production in the aqueous phase. As for HTL-AP, the metabolites were mainly VFAs. Thus, the acid content in the reactor would be lower, and the environment would be better for hydrogen production with the addition of crude glycerol. A similar improvement was also reported by Kanchanasuta et al. (2017), demonstrating that crude glycerol promoted the continuous generation of hydrogen, whereas the reaction stopped without glycerol addition [36].

After the first stage, the optimal hydrogen yield was 30.0 mL/g COD at a pH of 5.5. The corresponding second stage methane yield was 298.5 mL/g COD, which was 1.8 times higher than single stage HTL-AP fermentation. This result indicated that the enhanced first stage operation not only benefited the hydrogen generation, but more importantly it provided desirable intermediates for the second stage and improved the

overall energy conversion. As a valuable clean product, the enhanced hydrogen production can be used in several ways. On the one hand, hydrogen can be used during hydrocracking reactions for biocrude oil upgrading [57,58], then the continuous operation of HTL upgrading can be integrated with downstream two stage HTL-AP treatment as a whole system for renewable transportation fuel production. On the other hand, the enhanced hydrogen content could also be used for producing biohythane. The hydrogen to methane ratio of 0.1 in this study is within the optimal range of 0.1–0.2, which meets the requirements for biogas to serve as an alternative to replace industrial hythane gas [35].

3.2. Enhanced biomethane production in two stage co-fermentation

Fig. 3 shows the methane production from HTL-AP and crude glycerol co-fermentation. Comparing two stage with single stage (Fig. 3(d)), the optimal methane production was 207.4 mL/g COD with HTL-AP as the sole substrate (MA), resulting in a 25.5% higher methane production and a 24.2% (HA) shorter lag phase.

The effects of the initial pH value on the bioreactions extended from the first stage to the second stage methane production. Specifically, 9.5% and 16.3% higher methane yields were observed under a pH of 5.5 than that at a pH of 4.5 and 6.5 with HTL-AP as the sole substrate. While better promotions with crude glycerol addition were shown with a pH of 4.5 (Fig. 3(a)), leading to 7% and 21% higher methane yields than other pH conditions when the HTL-AP to glycerol ratio was 3:1. The optimal methane production was increased by 37.6%, 61.4%, and 69.3% compared to single stage HTL-AP fermentation as the crude glycerol content increased from 25% to 75% of the total substrate at a pH of 4.5. As for the methane production rate, a pH of 5.5 kept the lead for all HTL-AP groups with a 4.2%–36.5% higher rate than other pH groups.

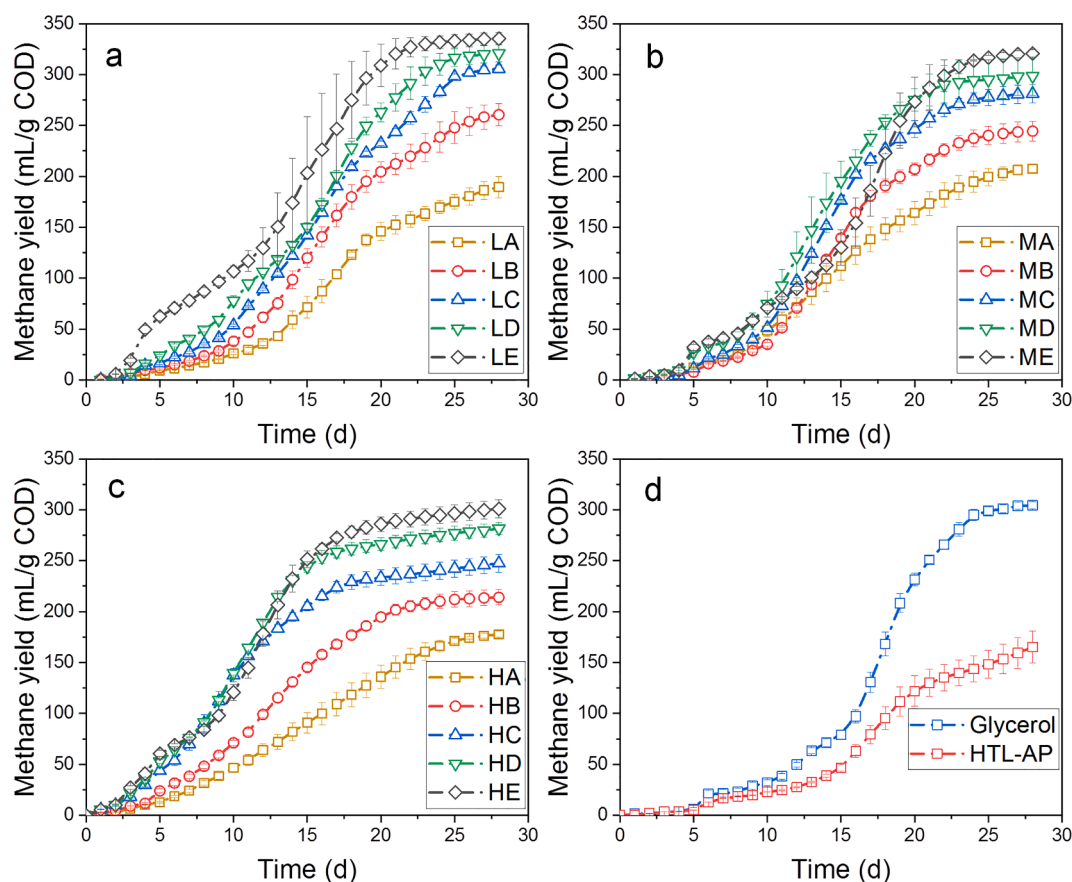


Fig. 3. Methane production from two stage co-fermentation at a pH of 4.5 (a), a pH of 5.5 (b), and a pH of 6.5 (c), as well as that from single stage fermentation at a pH of 6.1 (d).

Furthermore, a pH of 6.5 was most favorable for lag phase reduction. In particular, HC had the shortest start-up period of 3 days (Fig. 3, Table 4).

These results indicated that the methane yield, methane production rate, and lag phase were all affected by the initial pH value. Differences in the initial pH led to the formation of different intermediates and reaction conditions after the first stage, which in return leads to a difference in reaction kinetics during the second stage. Firstly, the higher initial pH of 6.5 provided a more suitable environment for the growth of methanogens and contributed to system stability, resulting in the shortest lag phase. Secondly, the lower pH of 4.5 led to an acidic shock, inhibiting the microbial metabolism and hydrogen production during the first stage. In return, the less consumed organics were conserved for the second stage which led to the highest methane yield. This result was consistent with the intermediates conversion in Fig. 2, signifying that no VFAs accumulation was observed for a pH of 4.5 during the first stage of co-fermentation. Notably, although the initial pH was carefully controlled, it gradually increased as the reaction continued. The reaction pH for all groups fell into the range of 7.1–7.7 for methanogenesis (Table A1) which coincided with the best pH range of 7.0–8.0 for most substrates and microorganisms [38]. Thirdly, the highest methane production rate was observed at a medium pH of 5.5. Comparing the intermediates conversion at this pH with a pH of 6.5 (Fig. 2), it's obvious that the concentration of VFAs was higher at a pH of 6.5 after the first stage, and the VFAs accumulation may lead to a slight inhibition of the growth of methanogens. Consistent with the higher hydrogen production, butyric acid was dominate in the effluent from the first stage at a pH of 5.5, and the concentration of propionic acid was much lower than that at a pH of 6.5. Propionate could lead to system instability [59], and its lower concentration at a pH of 5.5 helped alleviate this inhibition. These results indicated the continued impacts of the first stage on the second stage. Moreover, the initial pH was a key factor in determining the overall process kinetics, proving that the desired methane product can be obtained through initial pH adjustment.

A 17.9%–37.6% increase in methane yield was observed when the HTL-AP to glycerol ratio was 3:1 at a pH of 4.5–6.5 (Fig. 3 and Table 4) compared to single stage HTL-AP fermentation. As the glycerol content raised to 50% of the substrate, enhancement of the methane yield increased to 61.4%. The optimal yield of 320.8 mL/g COD was detected for a HTL-AP to crude glycerol ratio of 1:3 (LD), which was a 69.3% enhancement compared to the experiment conducted without crude glycerol addition (LA). Moreover, the methane production rate was enhanced by 1.39–2.17 times, and the optimal lag phase was shortened

by 48.1% with glycerol added as a co-substrate. Notably, a greater rise occurred when the crude glycerol content increased from 25% to 50% while the continued addition of glycerol to 75% did not provide as much improvement as before. This result indicated that a HTL-AP to crude glycerol ratio of 1:1 might be an optimal condition for two stage co-fermentation, which maximized the utilization of HTL-AP with enhanced biogas generation and energy conversion.

The general mechanism of crude glycerol addition during methane production was proposed in Fig. 4. Jensen et al. (2014) reported that co-fermentation did not facilitate synergy between the two co-substrates [37], suggesting that the enhancement may be attributed to the properties of glycerol and its influences on the reaction system. The pH buffering of crude glycerol mostly contributed to the first stage of hydrogen production as described previously. Furthermore, the effects as a nutrient balancer and a toxic diluent were more far-reaching during the second stage, although only a small amount of crude glycerol remained after the first stage, and the majority of the glycerol was converted into VFAs and alcohols such as 1,3-propanediol. On the one hand, with the reduced concentration of HTL-AP during co-fermentation with crude glycerol, the toxic compounds were also diluted. Toxic compounds in HTL-AP including nitrogen-containing organics and aromatic compounds could inhibit microbial growth and slow down the fermentation process, especially during the second stage. The toxicity of HTL-AP varies depending on the HTL feedstock. For example, Zheng et al. performed an anaerobic toxicity assay of the HTL-AP after the HTL of the algae *Spirulina* and found that 50% of the microbes were inhibited when the concentration of HTL-AP was 6% [11]. Thus, the utilization of a co-substrate was a feasible method for the dilution of toxic compounds, and it would have a greater strength for a highly toxic HTL-AP. On the other hand, crude glycerol could serve as a great carbon source to increase the carbon to nitrogen (C/N) ratio, reduce the ammonia concentration, release potential ammonia inhibition, and improve the conversion of organics [28]. The total nitrogen and ammonia nitrogen content of HTL-AP varies depending on the HTL feedstock. Although no obvious ammonia inhibition was present in this study, the ammonia content could be high for HTL-AP derived from other feedstock, especially algal biomass. A previous study reported that a total ammonia nitrogen concentration of 1700–1800 mg/L was completely inhibitory [60]. Previous studies reported that the ammonia concentration in HTL-AP from different algae could be higher than 4600 mg/L, and inhibition was observed [11,61,62]. Methanogens have been proven to be most sensitive to ammonia toxicity which causes cell growth to slow, resulting in methanogenesis inefficiency and even eventual shut down [60,63,64]. Thus, the C/N adjustment with crude glycerol addition could benefit the overall reaction performance during co-fermentation.

3.3. Organic conversion and energy recovery from two stage co-fermentation

Accompanied with an enhanced metabolism, the organic conversion of two stage co-fermentation was greatly promoted. To be specific, two stage operation increased the conversion rate by 12.6%–17.4% with HTL-AP as the sole substrate (A groups) from single stage HTL-AP fermentation (Fig. 5). Moreover, the addition of crude glycerol further enhanced the organic removal, leading to 30.5%, 48.6%, and 53.0% increases being observed when the crude glycerol content increased from 25% to 75%. Notably, a better organic conversion was achieved at a pH of 5.5 than a pH of 4.5 and 6.5, and this was consistent with the optimal hydrogen generation and methane production rate at the same condition. It is also worth noting that the actual conversion efficiencies (tested bar in Fig. 5) were higher than the calculated values (calculated bar in Fig. 5) in all groups, proving that the enhanced metabolism and biogas production was attributed to the synergistic effects of two stage operation, crude glycerol addition, and pH control.

With the enhanced hydrogen and methane generation, energy generation and recovery from co-fermentation was also promoted. The total

Table 4
Parameters of the modified Gompertz model fitting biomethane production.

Group	M _m (mL CH ₄ /g COD)	R _m (mL CH ₄ /g COD/d)	λ (d)	R ²
P	165	11.05	7.78	0.964
G	304	27.06	7.97	0.831
LA	189	13.21	7.14	0.950
LB	260	19.51	6.90	0.956
LC	305	24.91	7.00	0.943
LD	320	28.72	6.70	0.914
LE	335	34.38	5.72	0.872
MA	207	16.30	6.33	0.973
MB	244	22.71	6.95	0.954
MC	281	27.91	6.80	0.96
MD	298	29.93	6.14	0.953
ME	320	29.78	6.75	0.870
HA	178	13.10	5.90	0.956
HB	214	20.15	5.42	0.963
HC	247	20.44	3.06	0.998
HD	281	24.60	3.30	0.991
HE	301	27.46	3.97	0.972

M_m: maximum methane yield, R_m: maximum methane production rate, λ: lag phase. P and G were single stage HTL-AP and glycerol fermentations; L, M, and H groups were two stage co-fermentations at a pH of 4.5, 5.5, and 6.5; A, B, C, D, and E groups were two stage co-fermentation with a HTL-AP to crude glycerol ratio of 1:0, 3:1, 1:1, 1:3, and 0:1.

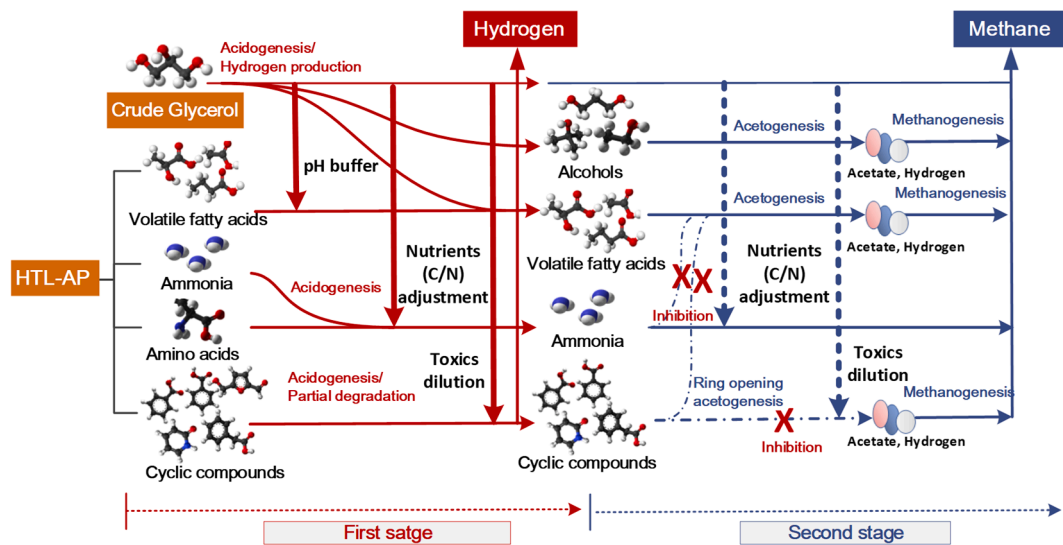


Fig. 4. Proposed theoretical reaction pathway for two stage co-fermentation of HTL-AP and crude glycerol. The dash dotted line shows the potential inhibition from ammonia and toxic cyclic compounds in HTL-AP; the solid red line shows the direct effects while the dotted blue line shows the indirect far-reaching influences of crude glycerol addition in two stages.

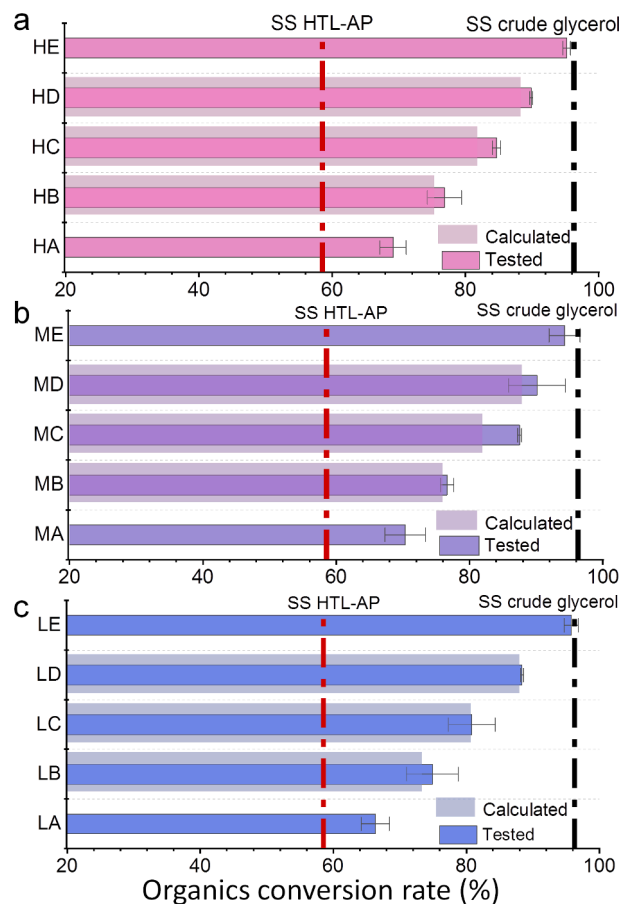


Fig. 5. Conversion of organics during two stage co-fermentation at a pH of 6.5 (a), a pH of 5.5 (b), and a pH of 4.5 (c). SS HTL-AP red dotted line: single stage HTL-AP digestion; SS crude glycerol black dotted line: single stage crude glycerol digestion; Tested bar: actual conversion rate; Calculated bar for group B, C, D: $B_{cal} = 0.75 \cdot A_{test} + 0.25 \cdot E_{test}$, $C_{cal} = 0.5 \cdot A_{test} + 0.5 \cdot E_{test}$, $D_{cal} = 0.25 \cdot A_{test} + 0.75 \cdot E_{test}$.

energy generation from biogas was calculated based on the heating values of hydrogen and methane. As shown in Fig. 6, 8.26 kJ/g COD energy generation was obtained with two stage HTL-AP fermentation (MA), which was 1.26 times higher than single stage fermentation (6.57

kJ/g COD). In addition, the optimal net energy production at a HTL-AP to crude glycerol ratio of 1:1 was 12.15 kJ/g COD (LC), which was improved by 85% from single stage fermentation. A similar result was reported by Kanchanasuta et al. (2017), noting that the energy

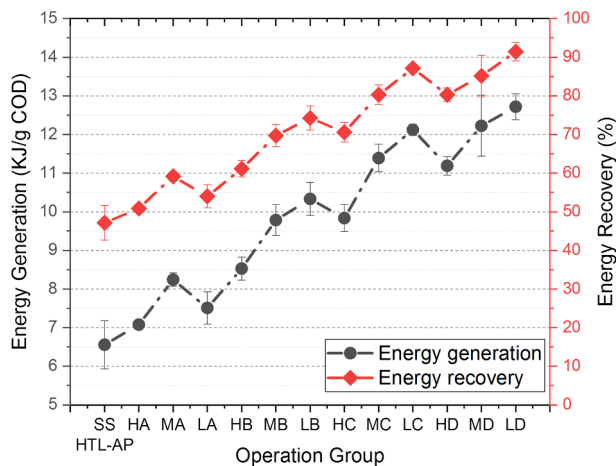


Fig. 6. Energy generation and energy recovery from two stage co-fermentation. SS HTL-AP shows the single stage HTL-AP fermentation; H, M, L represent the initial pH of 6.5, 5.5, and 4.5; A, B, C, D represent the HTL-AP to crude glycerol ratio of 1:0, 3:1, 1:1, and 1:3.

generation was improved by 6.2 and 1.6 times compared with single stage hydrogen and methane fermentation, respectively [36].

In terms of energy recovery, the optimal energy recovery rate with two stage HTL-AP fermentation was 59.3%, which was 12.1% higher than single stage fermentation (47.2%). This result was consistent with the fact that the theoretical energy recovery could be increased by 10–12% with two stage fermentation from single stage fermentation, as previously reported by Xia et al. (2016) [23]. With regard to co-fermentation with crude glycerol, the maximum energy recoveries were achieved at a pH of 4.5 which were 27.2%, 40.0%, and 44.4% higher than single stage fermentation when the content of crude glycerol increased from 25% to 75% (Fig. 6). These analyses of energy generation and energy recovery together indicated that two stage co-fermentation was a promising and energy efficient method to treat HTL-AP, and it had great potential for large scale application.

3.4. Application prospects

As a whole, the PCA and correlation plots in Fig. 7 depicted the synergistic effects of two stage operation, initial pH control, and crude glycerol addition on hydrogen and methane generation. Results indicated that the hydrogen yield was highly related to the initial pH value

and mix ratio. The methane yield and methane production rate were closely related to the HTL-AP to crude glycerol ratio, and the lag phase was more dependent on the stage operation. These results suggested that reaction parameters should be carefully controlled since an optimal condition could substantially promote digestion performance and efficiency. Notably, although crude glycerol addition always showed promotion effects in this study, its negative effects have also been reported and need to be prevented. Previous studies pointed out that glycerol could only facilitate digestion at a low concentration while inhibition due to the accumulation of VFAs would dominate as the concentration increased [37,65]. This finding also suggested that more investigations should be conducted in terms of the relationship between crude glycerol load, retention time, and their effects on a co-fermentation system before potential application of this methodology.

It is worth noting that two stage co-fermentation not only increased the methane yield, but it also greatly enhanced the hydrogen production, which was almost negligible during the traditional anaerobic digestion of HTL-AP. The greatly enhanced hydrogen generation provided another promising direction for two stage co-fermentation, because hydrogen can be used via hydrocracking for upgrading bio-crude oil, and methane could be used as heating source. Hence, post-HTL upgrading can be incorporated with the downstream HTL-AP treatment to establish a comprehensive system and provide a more sustainable alternative for converting wet biomass.

Compared with other strategies dealing with HTL-AP, two stage co-fermentation requires a low operating cost and condition. Large scale application has been demonstrated in previous studies with other types of feedstock. For example, Kopsahelis et al. (2018) performed a pilot scale two stage co-fermentation for 200 days, harvested a 90% higher methane yield than single stage fermentation, and obtained an additional 1.84 m³ hydrogen production per cubic meter of substrate [66]. Another pilot scale two stage co-fermentation enhanced the energy yield by 60%, and an additional 90 GWh gross energy yield can be achieved for a practical case [67]. Thus, the prospect of scaling up HTL-AP and crude glycerol co-fermentation is also very promising. Moreover, another critical advantage of two stage co-fermentation of HTL-AP and crude glycerol is the low solid content of the waste stream which makes it compatible with high-rate reactors (based on granules or biofilms). A techno-economic analysis conducted by Si et al. (2019) demonstrated that the minimum selling price of two stage HTL-AP fermentation in a high rate reactor was lower than gasoline under best and reference market conditions, indicating its huge commercial application potential [68]. Therefore, the industrial development of HTL-AP and crude glycerol two stage co-fermentation should be conducted and integrated with

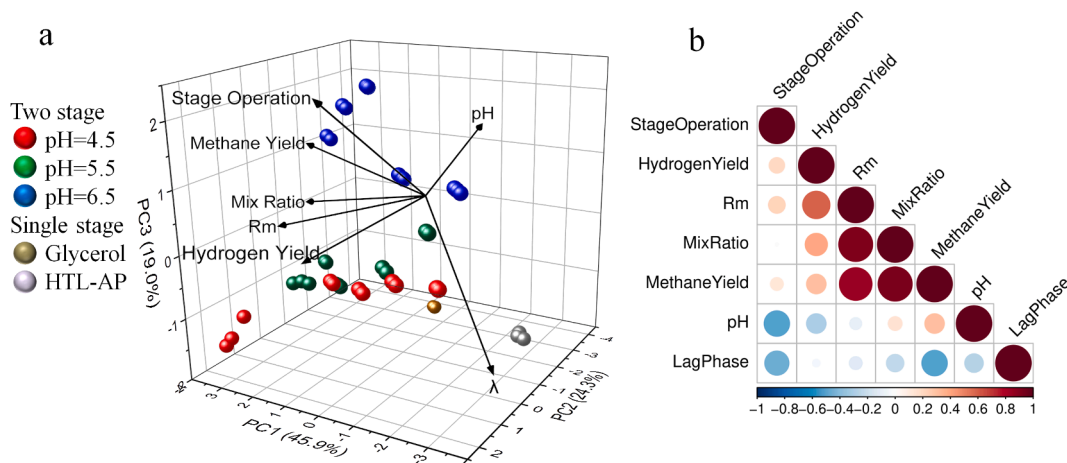


Fig. 7. PCA (a) and correlation plot (b) indicating the effects of stage operation, initial pH, and mix ratio on the hydrogen yield, methane yield, lag phase (λ), and gas production rate (R_m). In the PCA plot, positively correlated parameters are grouped together and negatively correlated parameters are positioned on opposite sides of the plot. In the correlation plot, positive correlations are in red and negative correlations are in blue.

upstream continuous HTL operations to facilitate waste treatment and maximize energy conversion.

4. Conclusion

This study demonstrated that two stage co-fermentation of HTL-AP and crude glycerol effectively improved the biohydrogen and biomethane production as well as the energy recovery. Two stage operation increased the biogas production by 25.5% in comparison with single stage HTL-AP fermentation. Further, the co-fermented crude glycerol served as a pH buffer, a nutrient balancer, and a toxic diluent that improved the system stability and organic conversion. A HTL-AP to crude glycerol ratio of 1:1 offered an 84.8% enhancement of the biogas yield in comparison with single stage fermentation. Moreover, the effects of initial pH values on two stage co-fermentation were investigated, and results showed that a pH of 5.5 was more beneficial to the conversion of organics and the hydrogen yield. The synergy of two stage operation, crude glycerol addition, and initial pH control made fermentation more effective, leading to a 48.6%, 84.9%, and 40.1% enhancement in organic removal, net energy generation, and energy recovery, respectively. This study demonstrated the opportunity to integrate two stage co-fermentation with HTL. Notably, the enhanced hydrogen could be used for hydrocracking during biocrude oil upgrading, and the methane could be utilized as the heating source. The promising future of two stage co-fermentation for larger scale applications was also revealed, which could facilitate waste treatment, enhance energy restoration, and provide a clean and sustainable fuel alternative.

CRediT authorship contribution statement

Zixin Wang: Investigation, Writing - original draft, Writing - review & editing. **Jamison Watson:** Writing - review & editing. **Tengfei Wang:** Writing - review & editing. **Shuqi Yi:** Investigation. **Buchun Si:** Supervision, Investigation, Writing - review & editing. **Yuanhui Zhang:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.enconman.2021.113855>.

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