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Nutrient recovery and biomass production by cultivating *Chlorella vulgaris* 1067 from four types of post-hydrothermal liquefaction wastewater

Li Zhang[1] · Haifeng Lu[1] · Yuanhui Zhang[1,2] · Baoming Li[1] · Zhidan Liu[1] · Na Duan[1] · Minsheng Liu[3]

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Abstract Cultivating microalgae in post-hydrothermal liquefaction wastewater (PHWW) can realize nutrient recovery, wastewater purification, and biomass production. This study investigated *Chlorella vulgaris* 1067 growth and nitrogen (N), phosphorous (P), and carbon (C) recovery from PHWW using 2×2 factorial experiments: two typical microalgae feedstocks (a low-lipid high-protein microalga, *Nannochloropsis* sp., and a high-lipid low-protein microalga, *Chlorella* sp.) for hydrothermal liquefaction (HTL) and two typical biocrude-aqueous separation methods (vacuum filtration and ethyl ether extraction). Results indicated that the feedstock and biocrude-aqueous separation method influence biomass production and nutrient recovery. PHWW from the high-lipid low-protein feedstock was advantageous to biomass production and nutrient recovery. *C. vulgaris* 1067 showed the best growth in 28.6 % PHWW obtained by vacuum filtration from *Chlorella* sp. Biomass production reached 1.44 g L^{-1} and N, P, and C recovery reached 209.25, 17.35, and 2588.00 mg L^{-1}, respectively. For the PHWW obtained from *Nannochloropsis* sp. and ethyl ether extraction, *C. vulgaris* 1067 showed better growth in 6.7 % PHWW. The biomass reached 0.67 g L^{-1} and N, P, and C recovery reached 147.19, 11.60, and 1150.00 mg L^{-1}, respectively. Regulating the pH value daily promoted the tolerance of microalgae to PHWW. Higher total organic carbon concentration, C/N ratio, volatile acid concentration, and lower nitrogen organic compound concentration in PHWW led to higher biomass and nutrient recovery. The ethyl ether extraction method for PHWW from low-lipid high-protein feedstock is one suggestion way to operate an environment-enhancing energy system efficiently.

Keywords Biocrude-aqueous separation methods · Biomass production · *Chlorella vulgaris* 1067 · Feedstock · Nitrogen, phosphorous, and carbon recovery · Post-hydrothermal liquefaction wastewater

✉ Haifeng Lu
hfcauedu@163.com

✉ Yuanhui Zhang
yzhang1@illinois.edu

[1] Laboratory of Environment-Enhancing Energy (E2E), College of Water Resource and Civil Engineering, China Agricultural University, Beijing 100083, China

[2] Department of Agricultural and Biological Engineering, University of Illinois at Urbana-Champaign, 1304 W Pennsylvania Avenue, Urbana, IL 61801, USA

[3] ENN Group Company, Huxiang Road, Economic and Technological Development District, Langfang 065001, China

Introduction

Post-hydrothermal liquefaction wastewater (PHWW) is generated from biocrude oil conversion via hydrothermal liquefaction (HTL). It retains most of the nitrogen (N), phosphorous (P), and a portion of the carbon (C) from the original feedstock (Jena et al. 2011; Zhou et al. 2013). These nutrients can be reused in the next cycle of microalgal biomass production for biocrude oil conversion via HTL. This new paradigm, called "environment-enhancing energy" (E^2-Energy), substantially amplifies microalgal biomass production and chemical conversion of biocrude oil, while simultaneously enhancing environmental quality (Yu et al. 2011; Zhou et al. 2013; Chen et al. 2014a, b). Throughout the whole paradigm, efficiently reusing N, P, and C by microalgae to produce HTL feedstock is a critical step in realizing the E^2-Energy scheme.

The potential of nutrient recovery from post-hydrothermal wastewater (PHWW) has had limited evaluation. Jena et al. (2011) characterized PHWW from *Arthrospira* (*Spirulina*)

platensis and evaluated its potential as a nutrient to culture *Chlorella minutissima*. Biller et al. (2012) also demonstrated the feasibility of using PHWW from different microalgae feedstocks and reaction conditions of HTL for nutrient recycling. Combing through a series of algal cultivation and HTL experiments, Zhou et al. (2011, 2013) successfully realized the running of an E^2-Energy system. All these studies found that it was possible to cultivate microalgae in diluted PHWW. However, the microalgal daily productivity was very low (0.0078–0.14 mg L^{-1} day^{-1}) and the PHWW was diluted at high ratios (20–600 times). This was largely attributed to the characteristics of PHWW. PHWW is rich in organic compounds and some inhibitors (such as dianhydromannitol, many nitrogen-containing compounds, and nickel), which at high concentrations become toxic to microalgae, resulting in substantial adverse effects on microalgal growth (Jena et al. 2011; Biller et al. 2012; Pham et al. 2013).

In previous research, most of the PHWW was separated from the oil stream by direct vacuum filtration (Jena et al. 2011; Zhou et al. 2013). Recently, it was discovered that light oil can be separated from the liquid phase using an organic solvent (Li et al. 2014). Organic compounds in PHWW that have similar polarity to the organic solvent will be extracted by the organic solvent, which can lead to a change of the characteristics of the PHWW. Consequently, microalgal growth and the N, P, and C recovery would both be influenced. Furthermore, the HTL feedstock affects the biocrude yield and its characteristics (Biller and Ross 2011; Lopez Barreiro et al. 2013), along with the distribution of elements in the HTL products. It is interesting that the biocrude-aqueous separation method and feedstock might allow microalgal growth and N, P, and C recovery to perform differently in PHWW. This could promote biomass production and nutrient recovery, which is worth investigating.

In this study, *Chlorella vulgaris* 1067 was cultivated in four types of PHWW using a 2×2 factorial experimental design: two typical microalgae feedstocks (a low-lipid high-protein microalga and a high-lipid low-protein microalga) were used for HTL and two typical biocrude-aqueous separation methods (vacuum filtration and ethyl ether extraction). The objectives of this work were (1) to evaluate *C. vulgaris* 1067 growth and nutrient recovery from the four types of PHWW and (2) to detail the effect of HTL feedstock and biocrude-aqueous separation on biomass production and N, P, and C recovery.

Materials and methods

Chlorella vulgaris 1067, a freshwater microalga, obtained from the Institute of Hydrobiology of the Chinese Academy of Science (Wuhan, China), was cultivated in a standard medium (BG-11) (Zhou et al. 2011).

The enrichment cultivation was carried out in 500-mL flasks. All flasks were placed in a light incubator with a light intensity of 170 μmol photons m^{-2} s^{-1} on a 12:12-h light:dark cycle at 26 °C. The cultures were shaken three times every day. All *C. vulgaris* 1067 used in the following experiments were in the logarithmic growth phase (approximately 4–6 days from the starting date).

Four types of PHWW

Four types of PHWW were prepared from two typical microalgae feedstock and two typical biocrude-aqueous separation methods. The two freshwater microalgae feedstocks were obtained from ENN Science and Technology Co., Ltd.: (1) *Nannochloropsis* sp. containing 14.1 % crude fat and 52.4 % protein (Li et al. 2014) was considered as a low-lipid high-protein microalga and was denoted by *Pr* (2); *Chlorella* sp. containing 59.9 % crude fat and 9.3 % protein was considered as a high-lipid low-protein microalga and denoted by *Ls* (Li et al. 2014). For the two biocrude-aqueous separation methods, separation by vacuum filtration was denoted by *F* (Fig. 1), and separation via ethyl ether extraction was denoted by *E* (Fig. 1). For the convenience, the four types of PHWW were abbreviated and defined as follows:

EPr The HTL feedstock was a low-lipid high-protein (*Pr*) microalga, *Nannochloropsis* sp., and the subsequent biocrude-aqueous was separated using ethyl ether extraction (*E*);

FPr The HTL feedstock was a low-lipid high-protein (*Pr*) microalga, *Nannochloropsis* sp., and the subsequent biocrude-aqueous was separated using vacuum filtration (*F*);

ELs The HTL feedstock was a high-lipid low-protein (*Ls*) microalga, *Chlorella* sp., and the subsequent biocrude-aqueous was separated using ethyl ether extraction (*E*);

FLs The HTL feedstock was a high-lipid low-protein (*Ls*) microalga, *Chlorella* sp., and the subsequent biocrude-aqueous was separated using vacuum filtration (*F*).

The reaction temperature of HTL was 300 °C, the reaction time was 60 min, and the solid content was 25 %. The concentration of total organic carbon (TOC), ammonia-nitrogen (NH_3-N), total nitrogen (TN), total phosphorous (TP), and pH values are shown in Table 1. The pH was measured with a pH meter (FE20, Mettler Toledo, Germany). The TOC was analyzed using a Torch Combustion TOC analyzer (TOC-VCPN, Shimadzu Co., Japan). The TN, TP, and NH_3-N were determined according to the American Public Health Association (APHA) standard method (Clesceri et al. 1998). The turbidity of the PHWW was determined using a HACH 2100N (USA).

The organic compounds of PHWW were determined using a gas chromatography–mass spectrometry (GC-MS) (Model

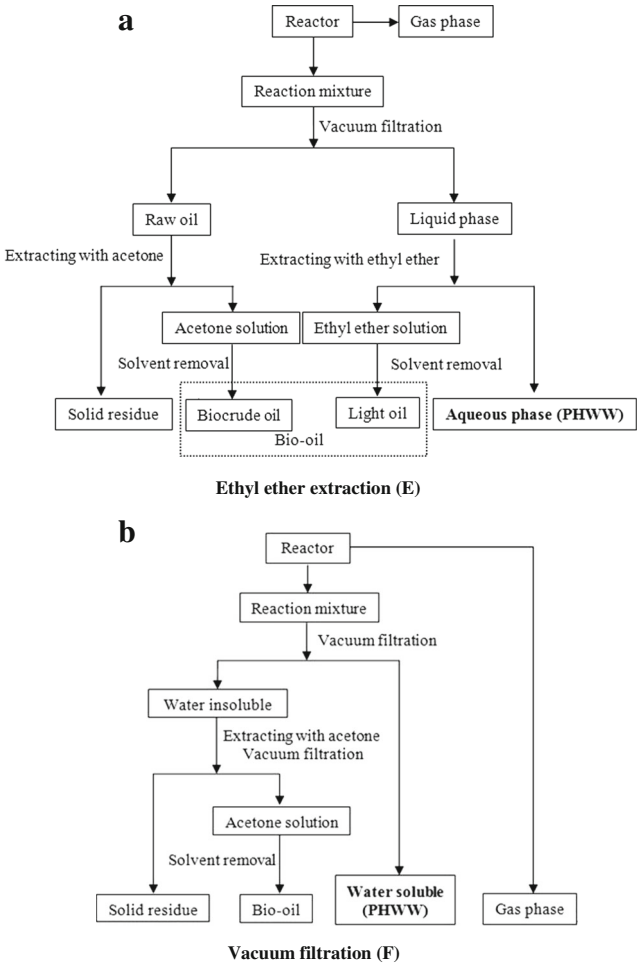

Fig. 1 Two separation methods of oil-aqueous. **a** Ethyl ether extraction (E). **b** Vacuum filtration (F)

QP2010, Shimadzu, Japan). The organic compounds in PHWW were extracted using a published method (Ren et al. 2006). The measurement conditions of the organic compounds within the PHWW were as follows: separation was achieved with a Varian DB-5 column (30 m×0.25 mm×0.25 μm). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. Dichloromethane extract (1 μL) was injected at 270 °C with a split ratio of 1:10. The column was initially set to 40 °C for 5 min, and then it was increased from 10 to 150 °C and held for 2 min; finally, it was increased from 5 to 270 °C with a hold time of 3 min.

Volatile fatty acids were analyzed by high-performance liquid chromatography (HPLC, 10A, Shimadzu, Japan) using a Synergi 4 μ Hydro-RP (Phenomenex) column. The mobile phase was 5 mM H_2SO_4 at a flow rate of 1 mL min^{-1} with a column temperature of 40 °C.

Table 1 Characteristics of PHWW

Parameters	EPr	FPr	ELs	FLs
pH	7.55–8.78	9.20	4.42–6.21	4.79
TOC (mg L^{-1})	80,000	35,480	52,180	14,626
NH_3-N (mg L^{-1})	4657	10,890	1636	1201
TN (mg L^{-1})	6867	12,888	2473	1729
TP (mg L^{-1})	453	968	16	82
Turbidity (NTU)	12	13	15	19

EPr is the PHWW separated by ethyl ether, and the HTL feedstock is a low-lipid high-protein microalga; FPr is the PHWW separated by vacuum filtration, and the HTL feedstock is a low-lipid high-protein microalga; ELs is the PHWW separated by ethyl ether, and the HTL feedstock is a high-lipid low-protein microalga; FLs is the PHWW separated by vacuum filtration, and the HTL feedstock is a high-lipid low-protein microalga

Experimental procedures

Batch experiments were conducted in 500-mL flasks to evaluate *C. vulgaris* 1067 growth and N, P, and C recovery from PHWW.

N is the primary nutrient for microalgae growth. Based on the initial TN concentration, the original PHWW was diluted with distilled water to achieve TN concentrations of the growth medium at four levels: 500, 250, 150, and 50 mg L^{-1}. Henceforth, these runs are referred to as TN500, TN250, TN150, and TN50 runs. BG-11 medium was the control. The volume of PHWW medium for each run was 400 mL. The pH of the PHWW was firstly adjusted to 7.1 by 1.0 M HCl or 1.0 M NaOH, and then each medium was sterilized at 121 °C for 30 min. When the sterilized medium was cooled to room temperature, *C. vulgaris* 1067 was inoculated into the media with 0.04–0.06 g L^{-1} dry cell weight.

The cultivation conditions were the same as for the microalgae strain preparation. The whole trial was carried out for 11 days. The pH of each run was re-adjusted daily to 7.0–7.5 with 1.0 M HCl or 1.0 M NaOH. The biomass of *C. vulgaris* 1067 was quantified by dry cell weight every day. For wastewater analysis, a 15-mL *C. vulgaris* 1067 suspension was sampled every 2 days. The samples were filtered through 0.45-μm membranes to remove microalgal cells, and the filtrate was stored at 4 °C for TOC, TN, and TP determination. All of the experiments were conducted in triplicate.

Analysis methods

The dry cell weight and daily productivity were used as indicators for comparing growth. The maximum specific growth rate (μ_{max}) and the half saturation coefficient (K_m) were used to investigate the growth potential and the potential utilization of nutrient, respectively. The removal ratio and removal quantity of N, P, and C were used to evaluate N, P, and C recovery.

Microalgal samples were filtered by a 0.22-μm pore size glass fiber filter (GTY1-BLQWΦ100 mm/0.22, Midwest Group, China) for dry cell weight measurement according to Lee and Shen (2004).

Daily productivity was calculated according to the following formula (Zhu et al. 2013):

$$\text{Daily productivity}\left(\text{g L}^{-1}\,\text{d}^{-1}\right) = \frac{\text{DCW}_\text{i}-\text{DCW}_0}{t_\text{i}-t_0} \quad (1)$$

where DCW_i and DCW_0 are the dry cell weight (g L^{-1}) at time t_i and t_0 (initial time), respectively.

The microalgae growth was described by the Monod model (Grady et al. 1999).

$$\mu\left(\text{day}^{-1}\right) = \mu_\text{max}\frac{C_\text{s}}{K_\text{m}+C_\text{s}} \quad (2)$$

where μ is the specific growth rate (day^{-1}); μ_max, the maximum specific growth rate (d^{-1}); C_s, the nutrient concentration (mg L^{-1}); and K_m, the half saturation coefficient (mg L^{-1}). μ_max represents the growth potential of the microorganism. In this study, the TN concentration was used for C_s. K_m was a measure of the affinity of algae for TN. Based on the kinetic function of μ with C_s, K_m and μ_max were calculated by the Lineweaver–Burk plot method (Lineweaver and Burk 1934; Lai et al. 2014).

The removal quantity was calculated using the following formula:

$$\text{Removal quantity}\left(\text{mg L}^{-1}\right) = C_0-C_\text{i} \quad (3)$$

where C_i and C_0 are the final and initial concentration, respectively, of TN, TP, and TOC (mg L^{-1}).

The removal ratio was calculated using the following formula:

$$\text{Removal ratio}\,(\%) = \frac{C_0\text{-}C_\text{i}}{C_0} \quad (4)$$

Statistical analysis

The data were statistically analyzed using one-way ANOVA (SPSS 17.0) based on the bottles as replicates (n=3). After checking the data for homoscedasticity and normal distribution of the variances, Duncan test was used for multiple average comparisons and to detect any differences between pairs of variables, at a significance level of $p<0.05$ and an extremely significance level of $p<0.01$.

Results

The optimal initial TN concentration for four types of PHWW to cultivate *C. vulgaris* 1067

C. vulgaris 1067 grew in all runs and better than in BG-11 medium (Fig. 2). This was different from other reports (Jena et al. 2011; Biller et al. 2012). The highest dry cell weight and daily productivity for each type of PHWW run were the EPr (TN500), FPr (TN250), ELs (TN500), and FLs (TN500) runs (Fig. 2 and Table 3). These four runs were selected for the subsequent comparative analysis of biomass production and nutrients recovery from the four types of PHWW.

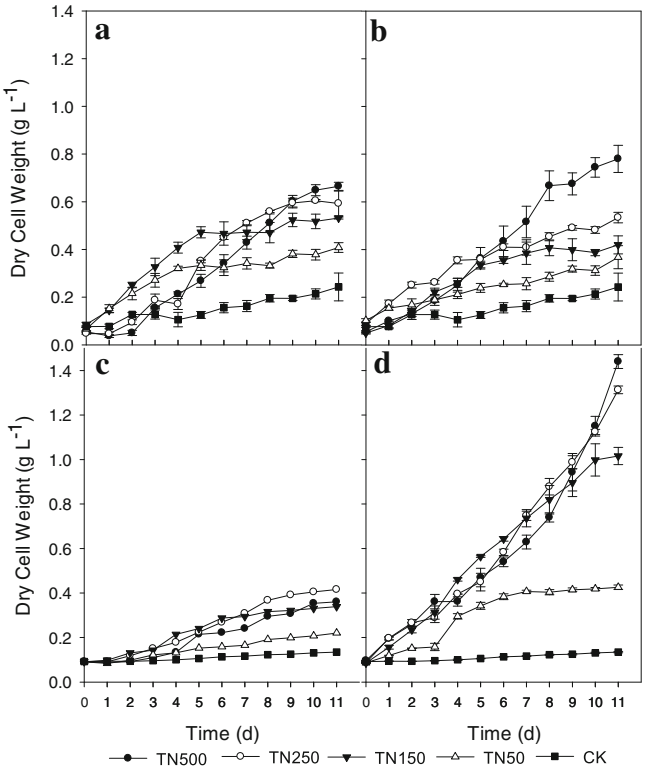

Fig. 2 Dry cell weight of *C. vulgaris* 1067 in diluted PHWW **a** FPr, **b** EPr, **c** ELs, and **d** FLs (n=3, ±sd). EPr is the PHWW separated by ethyl ether, and the HTL feedstock is a low-lipid high-protein microalga; FPr is the PHWW separated by vacuum filtration, and the HTL feedstock is a low-lipid high-protein microalga; ELs is the PHWW separated by ethyl ether, and the HTL feedstock is a high-lipid low-protein microalga; FLs is the PHWW separated by vacuum filtration, and the HTL feedstock is a high-lipid low-protein microalga

C. vulgaris 1067 showed high tolerance of TN and PHWW concentration. The initial PHWW concentration for FPr (TN250), EPr (TN500), ELs (TN500), and FLs (TN500) were 1.9, 6.7, 20.0, and 28.6 %, respectively, which were higher than that in previous reports, where it ranged from 0.2 to 1.2 % (Jena et al. 2011; Biller et al. 2012; Garcia Alba et al. 2013; Pham et al. 2013; Zhou et al. 2013). TOC and NH_3-N concentrations in this work were nearly 48 times and nine times higher than in the previous studies, respectively.

Biomass production from the four types of PHWW

With the same biocrude-aqueous separation method, *C. vulgaris* 1067 grew better in the PHWW from high-

lipid low-protein microalgae feedstock than that from low-lipid high-protein microalgae feedstock. μ_{max} ranged from high to low in the order FLs, ELs, EPr, and FPr (Table 2). Dry cell weight and daily productivity followed the order of FLs (TN500) > ELs (TN500) > EPr (TN500) > FPr (TN250). There was also a lag phase that appeared in the EPr and FPr runs (Fig. 2). These results showed that *C. vulgaris* 1067 might at first be inhibited. For FLs and ELs runs, there was no lag phase and biomass increased continuously till day 11. Hence, the TN concentration of PHWW from feedstock Ls for *C. vulgaris* 1067 cultivation is expected to be further improved. These results indicated that the PHWW from high-lipid low-protein microalgae feedstock (Ls) was more suitable for *C. vulgaris* 1067 growth than that from low-lipid high-protein microalgae feedstock (Pr).

Biocrude-aqueous separation methods also affected *C. vulgaris* 1067 growth. As shown in Table 2, for PHWW from feedstock Pr (low-lipid high-protein), dry cell weight and daily productivity in EPr (TN500) were both higher than in FPr (TN250) ($p<0.01$). For PHWW from feedstock Ls (high-lipid low-protein), dry cell weight and daily productivity of FLs (TN500) were both two times as much as that of ELs (TN500). Hence, for feedstock Pr, the PHWW separated by ethyl ether was more suitable for *C. vulgaris* 1067 growth. For feedstock Ls, the PHWW separated by vacuum filtration was more favorable for *C. vulgaris* 1067 growth.

N, P, and C recovery from the four types of PHWW

In the four types of PHWW, 47.49 to 7.05 % of TOC was recovered from PHWW (Fig. 3) by *C. vulgaris* 1067. N, P, and C recovered from PHWW that was generated from Ls were higher than that generated from Pr with the same biocrude-aqueous separation method. As shown in Fig. 3, the highest TN, TP, and TOC removal quantity appeared in the FLs (TN500) run ($p<0.01$), followed by the ELs (TN500) run and the EPr (TN500) run ($p<0.01$). The lowest TOC and TN removal quantities occurred in the FPr (TN250) run, and the lowest TP removal quantity occurred in the ELs (TN500) run.

For the PHWW generated from feedstock Pr, ethyl ether extraction was more suitable for N, P, and C recovery. As shown in Fig. 3, the removal quantities of N, P,

Table 2 Growth parameters in four PHWW (11 days, $n=3$, ±sd)

Runs	Dry cell weight (g L^{-1})	Daily productivity (g L^{-1} day^{-1})	Growth kinetic constant	
			μ_{max} (day^{-1})	K_m (mg L^{-1})
EPr			0.011	155.79
TN500	0.67 ± 0.017^{Aa}	0.055 ± 0.0015^{Aa}		
TN250	0.59 ± 0.052^{ABb}	0.050 ± 0.0045^{Ab}		
TN150	0.53 ± 0.0041^{Bc}	0.041 ± 0.00066^{Bc}		
TN50	0.41 ± 0.020^{Cd}	0.031 ± 0.0012^{Cd}		
FPr			0.0053	105.71
TN500	0.36 ± 0.0073^{Cc}	0.025 ± 0.00058^{Bb}		
TN250	0.42 ± 0.015^{Aa}	0.029 ± 0.0014^{Aa}		
TN150	0.34 ± 0.0090^{Bb}	0.023 ± 0.00082^{Cc}		
TN50	0.22 ± 0.0076^{Dd}	0.012 ± 0.00072^{Dd}		
ELs			0.014	290.60
TN500	0.78 ± 0.057^{Aa}	0.066 ± 0.0053^{Aa}		
TN250	0.53 ± 0.022^{Bb}	0.040 ± 0.0019^{Bb}		
TN150	0.42 ± 0.038^{BCc}	0.034 ± 0.0031^{Bc}		
TN50	0.37 ± 0.048^{Cc}	0.022 ± 0.00059^{Cd}		
FLs			0.23	3723.13
TN500	1.44 ± 0.031^{Aa}	0.12 ± 0.0028^{Aa}		
TN250	1.31 ± 0.017^{Bb}	0.11 ± 0.0016^{Bb}		
TN150	1.02 ± 0.039^{Cc}	0.085 ± 0.0035^{Cc}		
TN50	0.43 ± 0.010^{Dd}	0.031 ± 0.00076^{Dd}		

TN500, TN250, TN150, and TN50 are the runs of which the initial TN concentration are approximately 500, 250, 150, and 50 mg L^{-1}, respectively. μ_{max} and K_m are the maximum specific growth rate and the half saturation coefficient, respectively

Notation of superscripts: $^{A–D}$ Different capital superscripts within the same column represent extremely significant differences ($p<0.01$). $^{a–d}$ Different small superscripts within the same column indicate significant differences ($p<0.05$)

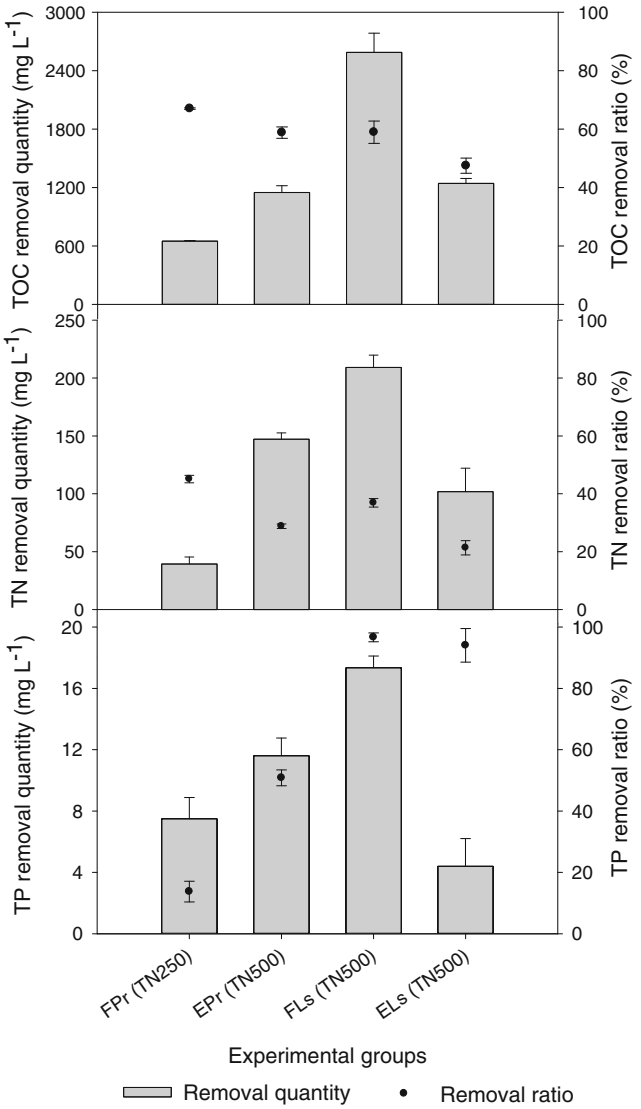

Fig. 3 Removal quantity and removal ratio of C, N, and P (11 days, $n=3$, ±sd)

and C in the EPr (TN500) run were higher than those in the FPr (TN250) run. For PHWW from feedstock Ls, vacuum filtration was more appropriate for N, P, and C recovery. The removal quantities of N, P, and C in the FLs (TN500) run were all twice as high as that of the ELs (TN500) run.

Discussion

The effect of feedstock and separation method on *C. vulgaris* 1067 growth

The results showed that *C. vulgaris* 1067 performed better in the PHWW from high-lipid low-protein microalgae feedstock than that from low-lipid high-protein microalgae feedstock, with the same biocrude-aqueous separation method. This result might be due to the different characteristics of the four different PHWW types.

Feedstock composition influences the element distribution and the existing forms of substance in the HTL biocrude oil and in the PHWW (Biller and Ross 2011), which might further influence the microalgae growth. The carbon and nitrogen proportions were very different among the various feedstocks, which led to different element distribution. In feedstock Ls, lipid provided the main portion (59.9 %), while in feedstock Pr, protein provided the largest portion (52.4 %). During HTL conversion processing, lipid was mainly converted into carbon-containing compounds (Chen et al. 2014a). On the other hand, protein was converted into nitrogen-containing compounds, and more protein in feedstock leads to more nitrogenous organic compounds in PHWW (Brown et al. 2010; Biller and Ross 2011; Duan and Savage 2011; Torri et al. 2012). Hence, the nitrogen compound proportion in PHWW from Pr was higher than that from Ls with the same biocrude-aqueous separation method. In addition, TOC took up over 95 % of the total carbon (TC), and the TOC removal was 67.0, 58.9, 47.4, and 59.0 % corresponding to FPr (TN250), EPr (TN500), ELs (TN500), and FLs (TN500), respectively (Fig. 3). Hence, *C. vulgaris* 1067 grew heterotrophically or mixotrophically in the PHWW medium. For this condition, higher carbon concentration runs might be more suitable for microalgae growth than the lower carbon concentration runs (Bhatnagar et al. 2011). The TOC concentration of ELs (TN500) was 34.3 % higher than that of EPr (TN500), and the TOC concentration of FLs (TN500) was 353.0 % higher than that of FPr (TN250) (Table 2). Therefore, higher biomass was obtained in PHWW from Ls than that from Pr. The C/N ratio in the PHWW resulted in different biomass production values as well. Xu et al. (2011) found that higher C/N ratio was beneficial for *Chlorella* to grow in a heterotrophic system. The initial C/N ratios of FLs (TN500), ELs (TN500), EPr (TN500), and FPr (TN250) were 7.7, 6.0, 4.0, and 3.8, respectively. Therefore, compared with FLs (TN500) and ELs (TN500), lack of carbon source was one possible reason for the lower biomass accumulation and

Table 3 The initial concentration of TOC, TN, NH_3-H, and TP in PHWW medium (mg L^{-1})

Runs	TOC	TN	TP	NH_3-H
EPr (TN500)	1954.00	494.23	20.98	310.51
FPr (TN250)	969.00	251.72	17.93	210.56
ELs (TN500)	2624.00	436.34	4.74	327.28
FLs (TN500)	4389.17	567.09	20.78	343.14

FPr (TN250) is the run of which the initial TN concentration is approximately 250 mg L^{-1}; EPr (TN500), ELs (TN500) and FLs (TN500) are the runs of which the initial TN concentration are approximately 500 mg L^{-1}

growth rate in EPr (TN500) and FPr (TN250). This suggests that supplying CO_2 to the PHWW from Pr might be advantageous for promoting biomass production (Table 3).

The microalgal growth might be inhibited or improved by other substances as well. During HTL processing, protein is converted into nitrogen-containing compounds, mainly including indole, pyrazine, pyridine, pyrrole, oxazoles, styrene, 2-phenylethanol, 1-phenylethanol, NH_3, and their derivatives (Brown et al. 2010; Biller and Ross 2011; Duan and Savage 2011; Torri et al. 2012). Some of these have potential toxicity to microalgae (Scragg 2006; Pham et al. 2013). Lipid is converted into carbon-containing compounds such as alkanes, alkenes, alkane halides, alkynes, short-chain fatty acids, and amides (Chen et al. 2014a). Some of these substances, such as short-chain fatty acids, are easily utilized by microalgae (Lee 2004). Previous research showed that, although NH_3-N might inhibit microalgal growth when nitrogen concentration is in the range of 850 to 1700 mg L^{-1}, no obvious effect was observed on the heterotrophic growth of *Chlorella* (Shi et al. 2000). In this work, the highest initial concentration of NH_3-N was 343.14 mg L^{-1} in FLs (TN500), and there was no inhibition of *C. vulgaris* 1067. Hence, the inhibition of NH_3-N might be omitted and other toxicities might have a negative effect on *C. vulgaris* 1067 growth. To further show the reduction, organic compounds in PHWW were determined by GC-MS in this work. Results showed that in PHWW from Pr, the products were ketone, alkanes, amides, pyridines, pyrroles, oxazoles, indole, and pyrazine, which might inhibit *C. vulgaris* 1067 growth (Scragg 2006; Pham et al. 2013). Hence, a lag phase might occur in the EPr and FPr runs rather than in the ELs and FLs runs. Volatile fatty acids (VFA) also appeared in all four types of PHWW (Table 4), and the VFA concentration was higher in the PHWW from feedstock Ls than that from feedstock Pr, which led to higher biomass in the FLs (TN500) and ELs (TN500) runs than that in the FPr (TN250) and EPr (TN500) runs.

The GC-MS results showed that fewer nitrogen–oxygen organic compounds were found in EPr (TN500) than in FPr (TN250). This indicated that some nitrogen–oxygen organic compounds might be extracted by ethyl ether. This is important for the operation of the E^2-Energy system because low-lipid high-protein microalgae are usually used in this system. Furthermore, the ethyl ether extraction method allowed light oil to be produced (Li et al. 2014), and thus, this method has been receiving more attention. Because there was a high lipid but low protein content in the feedstock Ls, the organic compounds were relatively simple in the PHWW from feedstock Ls compared to the Pr feedstock. Hence, the function of ethyl ether on reducing toxicity might be not very obvious for the PHWW from feedstock Ls. GC-MS determination showed the main organic compound composition was similar in ELs and FLs. But there was an obvious difference in the initial nutrient concentration. Based on the same initial TN concentration, the TOC concentration of FLs (TN500) was 67 % higher than that of ELs (TN500), and the TP concentration of FLs (TN500) was 338 % higher than that of ELs (TN500). Therefore, the higher carbon content was responsible for the increased biomass production in ELs (TN500).

Table 4 The concentration of volatile fatty acid (VFA) (mg L^{-1})

VFA	ELs	FLs	EPr	FPr
Formic acid	1.37	0.33	0	0.93
Lactic acid	20.33	0.65	0.87	0.64
Acetic acid	17.19	4.6	0.63	0.092
Succinic acid	1.3	1.68	0.052	0.32
Propionic acid	1.8	7.7	0.23	0.63
Butyric acid	1.38	0.096	0	0
Total VFA	43.37	15.06	1.78	2.61

The effect of feedstock and separation method on N, P, and C recovery

N, P, and C recovery was higher in PHWW generated from feedstock Ls than that from feedstock Pr. As discussed above, there were many nitrogenous organic compounds such as amides and ketones in the PHWW from feedstock Pr. These are irreversible competitive substances and are easily absorbed but difficult to be metabolized compared to non-nitrogenous small organic molecules (Alexander 1994). Thus, the nutrients that could be directly consumed by microalgae in EPr (TN500) and FPr (TN250) were relatively few, resulting in the specific growth rate reaching its highest level in a short time with a low K_m. For the PHWW from feedstock Ls, the VFA concentration was higher than that from Pr; therefore, *C. vulgaris* 1067 achieved higher biomass in the PHWW from feedstock Ls than in the PHWW from feedstock Pr, and N, P, and C recoveries were higher in FLs (TN500) and ELs (TN500) than those in FPr (TN250) and EPr (TN500).

For the PHWW from feedstock Pr, after ethyl ether extraction, some of the nitrogen-containing compounds might be removed. Hence, the inhibition by these substances in the PHWW to microalgae might be relieved, leading to higher N, P, and C removal in EPr (TN500) than those in FPr (TN250). The N/P ratio influenced the N, P, and C utilization as well. In a hetero-photoautotrophic system, the suitable N/P ratio for green microalgae growth should be in the range of 5:1 to 12:1, so that both of N and P could be efficiently utilized (Xin et al. 2010). The N/P ratios of ELs (TN500) and FLs (TN500) were 69:1 and 17:1, respectively. Hence, the depletion of TP led to the limitation of nitrogen uptake in ELs (TN500).

The effect of pH regulation on biomass production and N, P, and C recovery

C. vulgaris 1067 showed higher tolerance to PHWW in this work than in previous research. These effects could be caused by the daily pH regulation. pH affects the ratio of ammonium and ammonia. In aqueous solution, free ammonia exists in equilibrium with ammonium. When the pH increases, the equilibrium shifts towards ammonia (Azov and Goldman 1982). Compared with ammonium, ammonia is the main form of nitrogen which can be toxic to microalgae, as it easily passes through the cell membrane and binds with the thylakoids to inhibit photosynthesis (Abeliovich and Azov 1976). During microalgae growth, the pH of the medium will increases with time due to CO_2 uptake, which negatively affects microalgal growth. Hence, in this work, regulation of the pH to appropriately 7.0–7.5 daily allowed *C. vulgaris* 1067 to tolerate a high nitrogen concentration. This method might be more suitable for microalgae cultivation in PHWW than without pH regulation.

In conclusion, we investigated *C. vulgaris* 1067 growth in four types of PHWW and the influences of feedstocks and biocrude-aqueous separation methods on biomass production and nutrient recovery. The feedstock and biocrude-aqueous separation method influenced the characteristics of the PHWW, leading to different performance of *C. vulgaris* 1067 and nutrient recovery. Higher TOC concentration, C/N ratio and VFA concentration, and lower nitrogen organic compounds in PHWW were more suitable for biomass production and nutrient recovery. For the PHWW from Pr, through ethyl ether extraction, the potential toxicity of nitrogen organic compounds to microalgae might be relieved, which accelerated biomass production and nutrient recovery. These results can provide an effective guideline for effective E^2-Energy operation. As a fast-growing strain, low-lipid high-protein microalgae might be widely used in the future. Hence, further studies should be carried out on the effect of nitrogen organic compounds from PHWW on microalgae and how to relieve the toxicity. Additionally, the economic efficiency of using ethyl ether to separate light oil from the aqueous phase for nutrient recycling and algal cultivation should be carried out to evaluate its commercial application.

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