Effects of strain, nutrients concentration and inoculum size on microalgae culture for bioenergy from post hydrothermal liquefaction wastewater

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Abstract: Cultivating microalgae in post hydrothermal liquefaction wastewater (PHWW) offers many benefits, including nutrients recovery and reuse, wastewater purification and biomass production. However, the high nutrients concentration and toxic substances in PHWW undermine the efficiency of biomass production and nutrient recovery. This study aimed to investigate the effects of the microalgae strains, initial nutrients concentrations and inoculum sizes on biomass production and nutrient recovery using PHWW as the cultivation medium. Results indicated that both biomass production and nutrients recovery were successfully improved by using the screened microalgae strain at the desirable initial nutrient concentration with the suggested algae inoculum size. *Chlorella vulgaris* 1067 probably demonstrated the strongest tolerance ability among the five microalgae strains screened, and performed well in the diluted PHWW, of which initial TN concentration was approximately 500 mg/L. The desirable inoculum size was determined to be 0.103-0.135 g/L. The biomass daily productivity was increased by 15.67-fold (reached 0.13 g/(L·d)). With the above optimal conditions, high biomass production and nutrient recovery from the PHWW to produce microalgae biomass for bioenergy production were achieved.

Keywords: post hydrothermal liquefaction wastewater, microalgae strain screening, inoculum size, initial nutrient concentration, nutrient recovery, biomass production

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1 Introduction

Hydrothermal liquefaction (HTL) is a process in which wet biomass (such as biowaste and algae) is converted into biocrude oil under high-temperature and high-pressure[1,2]. This process generates a large amount of post hydrothermal liquefaction wastewater (PHWW) which can be used to produce microalgae[3,4]. The

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produced microalgae can be reused as a feedstock to produce biocrude oil conversion via HTL. The entire scheme, referred as the "Environment-Enhancing Energy" (E^2-Energy)[5], integrates wastewater treatment, algae biomass production and carbon capture, so that the economic value of bioenergy production can be maximized in an environmentally friendly approach. Multi-cycle reuse of the nutrient via microalgae production is a critical step to realize efficiently the E^2-Energy paradigm[5].

Previous studies have focused on the potential of using PHWW for microalgae cultivation[5-10]. However, the actual biomass productivity and nutrient utilization efficiency were unsatisfactory, with the microalgae daily productivity ranging from 0.0078 g/(L·d) to 0.045 g/(L·d). Although Alba et al.[6] and Selvaratnam et al.[7] got 0.19 g/(L·d) daily productivity, all the treatment are obtained in heavy diluted PHWW (the dilution factor was 20-600)[8-11]. This low efficiency might largely attribute to the characteristics of the raw PHWW. The concentrations of initial total organic carbon (TOC), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP) and ammonium (NH_3-N) in PHWW were 9060-15 123 mg/L, 80 000-128 000 mg/L, 3139-8136 mg/L, 280-3109 mg/L, and 4748-12 700 mg/L, respectively, which significantly exceeded the requirements for microalgae growth[6-10]. Moreover, some toxic substances in PHWW, such as dianhydromannitol, phenols and nitrogen-containing compounds (amides), can inhibit microalgae growth[9]. To ensure high biomass production, pretreatment for PHWW is necessary. This includes mitigation proper dilution or anaerobic biodigestion[12] and other methods. Until now, dilution is the most effective way to maintain adequate levels of available nutrient and while mitigate the inhibition effect of PHWW simultaneously[6-10].

It is important to obtain fast growing microalgae that are highly resistant to the toxins for the purpose of this research since microalgae activity and sensitivity intrinsically determine the quality and quantity of biomass accumulation and usually limit the removal rate of hazardous pollutants. It is known that PHWW contains many organic and hazardous substances and over 90% of total carbon in PHWW was in the form of organic carbon. Therefore, the microalgae strains that can utilize organic carbon and readily tolerant toxic substances are preferable. *Chlorella*, *Scenedesmus* and *Microcystis aeruginosa* have been reported to utilize organic carbon[13-17]. *Chlorella* sp. has the ability of highly resistant to inhibitors (e.g., high concentration of ammonium and COD) in wastewaters[18]. *M. aeruginosa* is a dominant species of cyanobacterial bloom in Taihu Lake in all seasons except winter[19]. In addition to glucose, *M. aeruginosa* can degrade many types of toxic substances, such as estradiol, phenol and amphetamine[20-22]. In PHWW, there are some substances that contain the same functional groups as amphetamines. Thus, it was assumed that *M. aeruginosa* can probably assimilate organic carbon and grow in PHWW. To explore other possibilities, five strains of microalgae including three strains of *Chlorella*, one strain of *Scenedesmus* sp. and *M. aeruginosa* were selected for this study.

Inoculum size of microalgae is another important factor in determining biomass productivity. The inoculum size significantly affects the lag phase, maximum specific growth rate, final biomass accumulation and the metabolite production of microalgae[23]. Markou et al.[24] reported that low inoculum sizes were more susceptible to ammonia inhibition than high inoculum size. Generally, the inoculum size positively correlates with the number of cells that will participate in reproduction, resulting in a higher biomass production[23]. However, excessive inoculum size will stress cell reproduction due to the limitation in nutrient and light[23]. Hence, a proper inoculum size can help to achieve high biomass production and nutrient recovery from PHWW.

This research aimed to: 1) identify the most productive strain among the five typical microalgae strains in PHWW under the given cultivation conditions; and 2) evaluate the influences of initial nutrients concentration and the inoculum size on algae biomass production and nutrients recovery. Furthermore, this study is expected to contribute to efficiently realize the E^2-Energy paradigm through improving biomass

production and nutrients recovery using the optimal results in aims 1) and 2).

2 Materials and methods

2.1 Characterization of PHWW

PHWW was obtained from a HTL experiment using *Nannochloropsis* sp. (provided by ENN Group Company) as the feedstock which contained 14.1% of lipid and 52.4% of protein. The detailed HTL experiment and its separation method of biocrude, PHWW and solid residue were described by Li et al.[25] The characteristics of raw PHWW used in this study are shown in Table 1.

Table 1 Characteristics of raw PHWW

Parameters	Values
pH	8.78
TOC/mg·L^{-1}	35 319.30
N/mg·L^{-1}	9891.80
TP/mg·L^{-1}	554.08
NH_3-N/mg·L^{-1}	8291.56

The PHWW was sampled by filtrating through 0.45 μm membranes to remove microalgae cells for TOC, TN and TP test. The pH was tested using a pH meter (FE20, Mettler Toledo Co., Inc., Germany). The TOC was analyzed using a Torch Combustion TOC analyzer (TOC-VCPN, Shimadzu Co., Tokyo, Japan). The TN, TP and NH_3-N concentrations were tested according to the APHA standard method[26].

2.2 Microalgae strain and preparation

The five microalgae strains were obtained from the Chinese Academic Institute of Hydrobiology (FACHB). They were *Chlorella vulgaris* 1067 (FACHB-1067), *Chlorella regularis var. minima* (FACHB-729), *Chlorella pyrenoidosa* (FACHB-10), *Scenedesmus quadricauda* (FACHB-507) and *Maeruginosa* (FACHB-315). All strains were cultured in a standard medium BG-11[27].

Microalgae cultivation was carried out in 500 mL flasks that were placed in an incubator with a light intensity of 170 μmol photons/m^2·s. Daily lighting schedule was 12 h on/off. The cultures were maintained at (26±1)°C. The cultures were manually shaken at least three times per day.

All microalgae strains used in the following experiments were inoculated at their logarithmic growth phase (approximately day 6).

2.3 Experimental procedures

2.3.1 Microalgae strain screening

The five microalgae strains were screened for the most productive strain using in the subsequent studies on the effects of initial nutrient and inoculum size. Firstly, PHWW was diluted with deionized water to a TN concentration approximately 250 mg/L. The pH of PHWW medium was adjusted to 7.1 by using 1.0 mol/L HCl or NaOH. PHWW medium was then sterilized at 121°C for 30 min. After PHWW medium was cooled down to room temperature, it was inoculated with approximately 0.12 g/L cell dry weight (CDW) microalgae in 500 mL PHWW medium. The experiments conditions were the same as the pure cultivation test above. The entire experiment lasted 12 d. Samples of microalgae biomass were collected daily to measure the CDW.

2.3.2 Initial nutrient concentration test

PHWW was diluted with deionized water to make sure the TN initial concentration were approximately 250 mg/L, 350 mg/L, 500 mg/L and 750 mg/L, respectively (Table 2). The logarithmic growth of *C vulgaris* 1067 was achieved from day 5 to day 7 without the phenomenon of wall build-up and clumping in this growth period. Hence, *C vulgaris* 1067 was inoculated at day 6. The inoculum size was approximately 0.08-0.13 g/L. The sterilization of PHWW media, the microalgae cultivation conditions and the cultivation period were the same as those described in microalgae strain screening test above.

Table 2 Characteristics of PHWW media used in experimental tests (mg/L, *n*=3, average±sd)

Items	Microalgae strain selection test	Initial nutrients concentration test				Inoculum size test
		TN250	TN350	TN500	TN750	
TOC	976.87±1.23	915.35±3.6	1507.33±51.47	1986.50±10.61	2063.23±2.80	2232.21±126.45
TN	247.41±3.62	273.76±4.76	399.30±12.58	516.64±10.66	749.00±0.00	550.09±31.88
TP	17.63±0.34	16.29±0.31	22.43±0.48	28.53±1.01	56.21±0.19	30.14±5.00
NH_3-N	207.04±11.80	228.84±9.03	334.45±10.38	432.52±8.71	627.83±7.35	461.02±3.25

Biomass CDW was measured daily. One milliliter PHWW was took out from the PHWW every day for the OD_{680} measurement. The biomass CDW was calculated by the OD_{680}-biomass curve as the following Equation:

$$Y_{Biomass}=0.3076X_{OD680}-0.0302 \quad (R^2=0.9972) \quad (1)$$

At the end of the test, a volume of 10 mL microalgae suspension was sampled by filtrating through 0.45 μm membranes to remove microalgae cells for the purpose of nutrient analysis in spent media.

2.3.3 Inoculum size test

The effect of inoculum size was evaluated using PHWW medium at the TN concentration of approximately 500 mg/L (Table 2). Diluted PHWW (1000 mL) was filled into 2 L flasks. *C vulgaris* 1067 was inoculated at day 6. The five levels of inoculum size were selected at 0.017 g/L, 0.060 g/L, 0.103 g/L, 0.135 g/L and 0.160 g/L, which were henceforth referred to as IN1, IN2, IN3, IN4 and IN5, respectively. The sterilization of PHWW media, the microalgae cultivation conditions and cultivation period were the same as those described in microalgae strain screening test above. Microalgae biomass CDW was measured daily. The spent medium samples were collected every other day for the purpose of nutrients analysis.

2.4 Analysis methods and data processing

The CDW, daily productivity (for 12 d) and maximum specific growth rate (μ_{max}) were used as the indicators for microalgae growth. The removal quantity, removal ratio and the cellular consumption of nutrients (Nu/B) were used to evaluate the N, P and C recovery, respectively, from PHWW.

The CDW was measured according to the method in publication[28]. Algae samples were filtered by 1.5 μm pore size glass fiber filter (Whatman 934AH) and subsequently dried at 60°C until a constant weight.

Daily productivity (g/L·d) was calculated according to the following formula[10]:

$$\text{Daily productivity} = \frac{CDW_i - CDW_0}{t_i - t_0} \quad (2)$$

where, CDW_i and CDW_0 are the final and initial concentrations of CDW, g/L, respectively; t_i and t_0 are the final and initial time, d.

The microalgae growth was described using the Monod model.

$$\mu = \mu_{max}\frac{C_s}{K_m + C_s} \quad (3)$$

where, μ, μ_{max}, C_s and K_m are the specific growth rate, d^{-1}; maximum specific growth rate, d^{-1}; TN concentration, g/L; and half saturation coefficient, g/L, respectively. μ_{max} stands for growth potential. Based on the kinetic function of μ with C_s, K_m and μ_{max} were calculated by regression according to the Line weaver-Burk plot[29,30].

Removal quantity (mg/L) and removal ratio were calculated using Equations (4) and (5), respectively:

$$\text{Removal quantity} = C_0 - C_i \quad (4)$$

$$\text{Removal ratio} = \frac{C_0 - C_i}{C_0} \quad (5)$$

where, C_i and C_0 are the final and initial concentrations of the nutrients (TOC, TN and TP), respectively. The removal quantity that was showed in Section 3.2 was calculated by measuring the initial (at 0 day) and finial (at 12th day) concentration of TOC, TN and TP, respectively.

The Nu/B (g/g) was calculated according to the following Equation[31]:

$$Nu/B = \frac{C_0 - C_i}{CDW_i - CDW_0} \quad (6)$$

where, *Nu* stands for the nutrients; *B* stands for the biomass.

2.5 Statistical analysis

Each experiment was carried out in triplicate. All the results are presented as the mean values with standard deviations. Based on the bottles as replicates (n=3), one-way ANOVA (SPSS 17.0) and Duncan test were 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 $p<0.01$.

3 Results

3.1 Microalgae strain screening

Figure 1 showed the growth trend of the five microalgae strains in diluted PHWW. *C. vulgaris* 1067 yielded the highest biomass. The daily productivity ranged from high to low was *C. vulgaris* 1067 (0.031 g/(L·d)), *M. aeruginosa* (0.024 g/(L·d)), *S quadricauda*

(0.0071 g/(L·d)), *C. pyrenoidosa* (0.0041 g/(L·d)) and *C. regularis var. minima* (0.0022 g/(L·d)). After the lag phase about 2 d, the CDW of *C vulgaris* 1067 reached 0.24 g/L and then maintained the digits until 10 d. No lag phase appeared in *M. aeruginosa*, but the CDW was always lower than that of *C vulgaris* 1067. There was either no lag phase for *S. quadricauda* and *C. pyrenoidosa*, but CDW of them both were much lower compared to *M. aeruginosa*. Considering the biomass daily productivity, *C. vulgaris* 1067 was chosen as the superior microalgae for biomass production from PHWW.

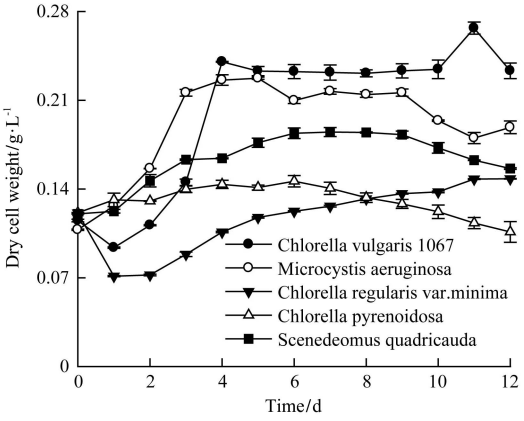


Figure 1 Dynamic profiles for five microalgae strains

Table 3 Productivity and maximum time of microalgae strains (*n*=3, average±sd), 11 d

Microalgae strains	Daily productivity[a] /g·L^{-1}·d^{-1}	Maximun time[b] /d
Chlorella vulgaris 1067	0.031	4
Microcystis aeruginosa	0.024	5
Scenedeomus quadricauda	0.0071	6
Chlorella pyrenoidosa	0.0041	6
Chlorella regularisvar. minima	0.0022	12

Note: [a] Daily productivity of Max. time. All values were reported as average of three replications, standard deviations ranged from 0.8 to 0.9; [b] The time of maximum biomass.

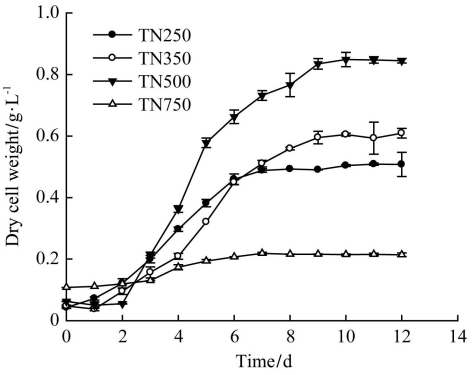
C. vulgaris and *M. aeruginosa* performed better than the other three microalgae strains on growth. That might be caused by the different physiological and biochemical characteristics of different types of microalgae. There were more literature reviews on *C. vulgaris* and *M. aeruginosa* to degrade organic substances and pollutants than the other three microalgae strains[14,32,33]. *C. vulgaris* and *M. aeruginosa* could consume some micro-molecular organics (e.g., glucose, acetate, glutamate and lactate) and some toxic or macromolecular organics (e.g., azo compounds, phenol, estradiol and amphetamine)[20-22,28]. This phenomenon might indicate that the utilization range of substances for *C. vulgaris* and *M. aeruginosa* was wider and the tolerance to organic substances was stronger than the other three microalgae strains. PHWW is a very complicated wastewater which contains abundant organic matters and toxic substances[8,33]. The strong adaptation of *C. vulgaris* 1067 and *M. aeruginosa* to different kinds of organic substances might lead to their better performance than the other microalgae in PHWW.

In this study, the day alternates with night external light condition (12 h on/off), and the organic substances removal was very high (the TOC removal ratio was above 50%). This showed that in PHWW, mixotrophy might be the most plausible growth mode for the five microalgae to growth. The characteristics of mixotrophic mode for these microalgae are very different. It is reported that nitrogen concentration of 850-2000 mg/L did not markedly affect the mixotrophic growth of *Chlorella*[28,34]. In this test, the initial concentration of TN and NH_3-N was 247.41 mg/L and 207.04 mg/L, respectively, which did not have adversely affected on *C. vulgaris* 1067. The optimal concentration of nitric nitrogen for *M. aeruginosa* growth was 32.0-64.0 mg/L[35] in a mixotrophic culture. In PHWW, the initial concentration of nitric nitrogen was below 2 mg/L, which was not in the optimal concentration of nitric nitrogen for the mixotrophic growth of *M. aeruginosa*. Biller and Ross[33] also found that in their study, *Scenedesmus* sp. can cope with a lower nutrient availability than *C. vulgaris*. Under mixotrophic mode, the maximum specific growth rate of *C. vulgaris* (0.198 d^{-1}) was higher than that of *Sacutus* (0.048 d^{-1})[28]. In this work, *Squadricauda* belongs to *Scenedesmus*, which might have similar characteristics of metabolism mode to *S. acutus*. Hence, *C. vulgaris* 1067 demonstrated the best performance and was thus selected for the subsequent experiments to evaluate the effects of initial nutrient concentrations and inoculum size on biomass production and nutrients recovery.

3.2 Effect of initial nutrient levels on biomass production and nutrient recovery

Table 2 showed that *C. vulgaris* 1067 tolerated TN

concentration ranging from 273.76 mg/L to 749.00 mg/L. The initial PHWW concentration level influenced the final biomass accumulation and daily productivity of *C. vulgaris* 1067. In addition, TN500 run was a threshold run. Higher TN concentration was benefit for *C. vulgaris* 1067 growth when the initial TN concentration was below 500 mg/L. The highest CDW and daily productivity occurred in TN500 run (Figure 2 and Table 4), in which the accumulative biomass and daily productivity reached 0.84 g/L and 0.065 g/(L·d) on the 11th day, respectively. However, when the TN concentration of PHWW was above 500 mg/L, especially was approximately at 750 mg/L, the final biomass accumulation was much lower than that of the other runs.



Note: The TN concentration of TN250, TN350, TN500 and TN750 was 273.76 mg/L, 399.30 mg/L, 516.64 mg/L and 749.00 mg/L, respectively.

Figure 2 Biomass formation of *C. vulgaris* 1067 at different nutrient concentration levels

Table 4 Daily productivity and Nu/B at different nutrient concentration levels, 11d (*n*=3, average±sd)

Runs	Daily productivity /g·L^{-1}·d^{-1}	Removal quantity/mg·L^{-1}			Nu/B/g·g^{-1}		
		TOC	TN	TP	TOC	TN	TP
TN250	0.038±0.0028^{Cc}	534.60±12.62^{Cc}	66.99±5.14^{Cc}	7.89±0.18^{Bb}	1.13±0.12^{Cc}	0.14±0.028^{Bc}	0.017±0.0018^{Bb}
TN350	0.047±0.0011^{Bb}	876.23±28.19^{Bb}	115.02±9.19^{Bb}	12.35±0.55^{Aa}	1.52±0.00045^{Bb}	0.21±0.0024^{Ab}	0.021±0.00031^{Aa}
TN500	0.065±0.00022^{Aa}	1150.00±64.80^{Aa}	157.06±7.36^{Aa}	12.02±1.16^{Aa}	1.75±0.070^{Aa}	0.24±0.0059^{Aa}	0.018±0.00078^{Bb}
TN750	0.0086±0.000068^{Dd}	51.90±2.48^{Dd}	14.29±1.20^{Dd}	1.15±0.16^{Cc}	0.48±0.0061^{Dd}	0.13±0.0030^{Bc}	0.013±0.00047^{Cc}

Note: $^{A-D}$ 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). Different alphabet stands for the extremely significant or significant differences; same alphabet means the differences were not significant. Nu/B is the cellular consumption of nutrients.

The TN concentration of TN250, TN350, TN500 and TN750 was 273.76, 399.30, 516.64 and 749.00 mg/L, respectively.

The initial PHWW concentration level also influenced the growth trend of *C. vulgaris* 1067. Except TN 250 run, all the other runs showed lag growth phases. The lag phase time was prolonged as the levels of PHWW concentration increased. The logarithmic growth phase meant the totally adaptation to the environment. During this phase, the growth rate of microalgae is the highest. In addition, microalgae are always harvested at the end of logarithmic growth phase. In this test, the logarithmic was the shortest for TN750 run than the other runs.

The different performances of final biomass accumulation and growth trends of *C. vulgaris* 1067 in different nutrients levels could be caused by the characteristics of PHWW. PHWW is a kind of complicated organic industrial wastewater. On the one hand, it contains some micro molecular substances, such as volatile fatty acids (formic acid, lactate, acetate, succinic acid, propionic acid and butyric acid) and ethanol[36], which can be absorbed as nutrients for microalgae. On the other hand, some toxic substances, such as alerolactam, 2-piperidone, 2,2,6,6-tetramethyl-4-piperidone, and 1-methyl-2pyrrolidinone[9] in PHWW can cause inhibition effect to microalgae. They are inhibitor for microalgae. Higher concentration of PHWW contains higher concentration of inhibitors, which lead to negative performance of biomass production. Therefore, the lag phase should appeared at the beginning of the growth trend, and the higher the initial concentration levels, the more time *C. vulgaris* 1067 needed to adapt the environment, which lead to the prolonged lag phase time. However, for TN250 run, *C. vulgaris* 1067 directly entered log phase without adaptation to the PHWW. That might be caused by the low inhibitor concentration levels in this run. Under this concentration, the concentration of toxic substances might have no inhibition effect on *C. vulgaris* 1067. It seems that in Jena et al.[9] and Biller and Ross's reports[33], there was no lag phase appeared as well, in which the TN concentration of PHWW were 162 mg/L and 17.22 mg/L, respectively. These results also provided that low

PHWW concentration can relieve the inadaptation of *C. vulgaris* 1067 growth to PHWW. Although the logarithmic growth phase was shorter in TN500 run than that in TN350 run, the nutrients concentration (volatile fatty acids) in TN500 run was higher than that in TN350 run, which led to the highest biomass accumulation and fast growth rate of *C. vulgaris* 1067 in TN500 run.

After the adaptation to the environment, *C. vulgaris* 1067 entered into the logarithmic phase to growth. Higher nutrients concentration leads to higher biomass production. That meant, higher PHWW concentration run provide more nutrients for microalgae, which lead to higher biomass production. However, as it was described above, the risk of inhibition to microalgae might be promoted with the organic substances level increasing. When the TN concentration was around 750 mg/L, the biomass production was the lowest and there was obvious inhibition effect to *C. vulgaris* 1067 appeared with the longest lag phase and shortest logarithmic growth phase time. That meant in TN 750 run, the adverse affection of the organic substances (especially the toxic substances) to microalgae was stronger than in other runs. Pham et al.[9] investigated that *C. protothecoides* can degrade the organic compounds and when PHWW was increased to 11.3% (the concentration of *δ*-valerolactam, *ε*-caprolactam, 1-methyl-2-pyrrolidinone and 2-pyrrolidinone were 15.7 mg/L, 1.1 mg/L, 0.2 mg/L and 0.09 mg/L, respectively), algae growth was inhibited. In this work, valerolactam, 2-piperidone, 2,2,6,6-tetramethyl-4-piperidone, and 1-methyl-2pyrrolidinone were found in PHWW. The initial characteristics of PHWW in this work were very similar to that in Pham et al.'s work[9]. After dilution, the PHWW concentration of TN750 was 7.6%, in which the above toxic substances concentration was very close to the concentration of Pham et al.'s results[9]. Therefore, the inhibition effect was obvious in TN750 run.

In this work, the removal quantity of TOC, TN and TP represents the absolute amount of nutrient consumed by the microalgae. Apparently, a high removal quantity is desirable from the nutrient recovery point of view. As shown in Table 4, the highest removal quantity of TOC and TN all occurred in TN500 run (p<0.01). Especially, the highest removal quantities of TOC and TN were 1.3-22.2 times, 1.3-11.0 times of the other runs, respectively. The highest TP removal quantity appeared in TN350 run, which was significantly higher compared to the other runs (p<0.01). Nu/B presents conversion of nutrients to algae cells. The higher value of Nu/B presents that the more nutrient is consumed by microalgae based on a certain CDW, and it is more advantageous to the efficient nutrient cycle. The highest Nu/B of TOC and TN also occurred in TN500 run (p<0.05).

The highest of Nu/B and nutrient removal quantity all matched the biomass production. It was largely attributed to the algae cell activity. It was well known that metabolic activity of algae cells is most intense and cell reproduction exceeds cell death in logarithmic growth phase. Obviously, microalgae cells maintained a long logarithmic growth phase in TN500 run. Therefore, stronger activity of algae cells caused the more efficient biomass production and nutrient recovery in TN500 run than those of the other runs.

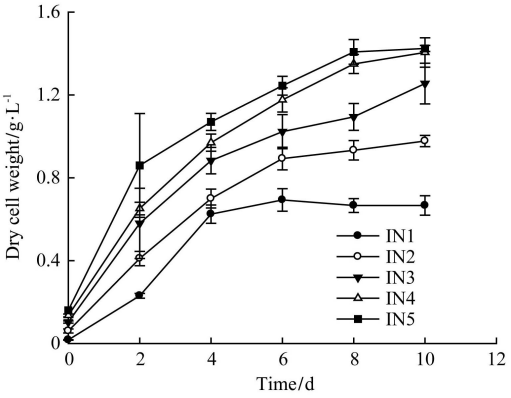
In addition, it is worth of noting that *C. vulgaris* 1067 can tolerate higher concentrations of PHWW and accumulate more biomass in this study. In literature reports that the initial TOC, NH_3-N and TP in diluted PHWW were 90.60-137.76 mg/L, 25.40-58.7 mg/L and 1.59-31.09 mg/L, respectively[6,8-10,33]. In this work, the TOC, NH_3-N and TP concentration of PHWW in TN500 run was higher than that in literature reports, especially, the highest initial TOC and NH_3-N concentrations in the diluted PHWW (TN750) were 15-23 times and 11-21 times higher, respectively, than those in literatures. The daily productivity of TN500 run was also higher than most of the other reports (0.013-0.044 g/L·d) presented[8-10], resulting a higher nutrient removal quantity. These results were largely due to the biocrude-aqueous separation method, which affects the elements distribution and productions composition from HTL[12,27]. In this work, the PHWW was separated by ethyl ether from light oil according to the published biocrude-aqueous separation method[12]. Compared with the report method where the PHWW was obtained from

the direct vacuum filtration of biocrude-aqueous mixture[7,8], ethyl ether extracted some nitrogen-oxygen organic compounds out along with light oil, which might alleviate the toxicity of PHWW and positively influence algae growth. On the other hand, the concentrations of TOC, TN and TP in PHWW (Table 1) in this study were in accordance with the data in the literature reports[5-10]. Therefore, the biocrude-aqueous separation method did not affect the concentration of TOC, TN and TP of PHWW, but the composition of organic compounds in PHWW, which caused *C. vulgaris* 1067 could tolerance higher PHWW concentration and grew better. For the multi-cycle nutrient in E^2-Energy system, it is critical to choose the biocrude-aqueous separation method.

C. vulgaris 1067 could realize growth under the TN concentration as high as 749.0 mg/L. However, the microalgae were not domesticated in PHWW culture medium in this study. If a strain has been domesticated in PHWW media, the initial nutrient content could be higher than 749.0 mg/L, which could lead to a further increase in nutrient recovery and biomass production. This hypothesis remains to be further investigated.

3.3 Effect of inoculum size on biomass production and nutrient recovery

Experimental results are shown in Figure 3 and Table 5 indicate that the high inoculum sizes (IN3, IN4 and IN5 runs) were more favorable for biomass production than the low inoculum sizes (IN1 and IN2 runs). *C. vulgaris* 1067 performed better in high inoculum sizes (IN3, IN4 and IN5 runs) than in low inoculum sizes (IN1 and IN2 runs). Moreover, under the same nutrients concentration level, higher microalgae dosage shorted the arrival time to stationary phase. For IN4 and IN5 runs, they reached the stationary phase at the 8th day while for IN3, there was no stationary phase appeared during the test time. For IN1 and IN2 runs, they reached the stationary phase at the 4th and 6th day, respectively. The value of μ_{max} presents the growth potential of microalgae. As shown in Table 5, the μ_{max} ranged from high to low were: IN4 (0.061 d^{-1}), IN3 (0.045 d^{-1}), IN5 (0.034 d^{-1}), IN2 (0.028 d^{-1}), IN1 (0.0081 d^{-1}). It indicates that *C. vulgaris* 1067 had maintained higher potential growth activity in the runs of high inoculums size (IN3, IN4 and IN5) than that in low inoculum size (IN1 and IN2). However, microalgae showed lower growth potential in IN5 run, compared to that in IN3 and IN4 runs. Therefore, the suitable range of inoculum size for biomass production was between 0.103 g/L and 0.135 g/L as in the runs of IN3 and IN4. In addition, the desirable inoculum size in this test increased biomass productivity by 2-fold compared to TN500 in the initial nutrient levels test.



Note: The inoculum sizes of IN1, IN2, IN3, IN4 and IN5 was 0.017 g/L, 0.060 g/L, 0.103 g/L, 0.135 g/L and 0.160 g/L, respectively

Figure 3 Biomass formations of *C. vulgaris* 1067 at different inoculum sizes

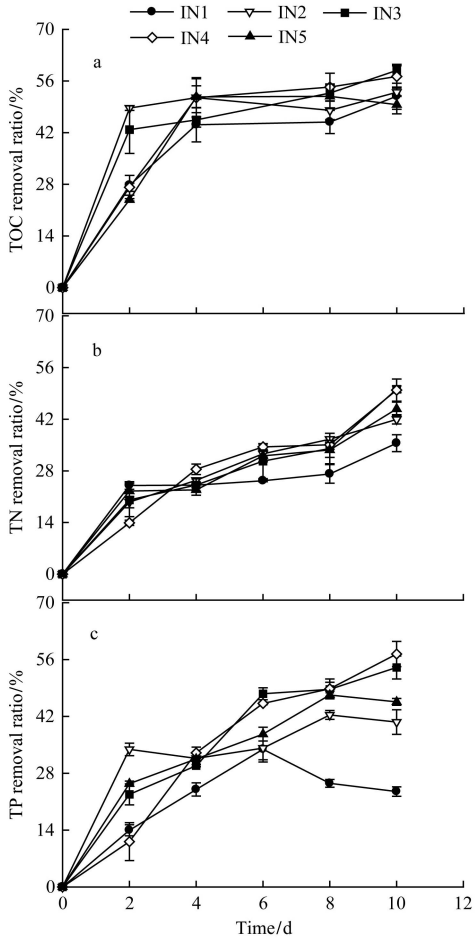
Table 5 Growth parameters of *Chlorella vulgaris* 1067 for different inoculum sizes (n=3, average±sd)

Runs	IN1	IN2	IN3	IN4	IN5
Daily productivity /g·L^{-1}·d^{-1}	0.068± 0.00^{Aa}	0.090± 0.0011^{Bb}	0.12± 0.0013^{Cc}	0.11± 0.0012^{Dd}	0.13± 0.0012^{Ee}
μ_{max} /d^{-1}	0.0081	0.028	0.045	0.061	0.034
K_m	142.01	22.73	25.07	28.22	26.63

Note: $^{A-E}$ Different capital superscripts within the same row represent extremely significant differences (p<0.01); $^{a-e}$ Different small superscripts within the same row indicate significant differences (p<0.05). Different alphabet stands for the extremely significant or significant differences; same alphabet means the differences were no significant. IN1, IN2, IN3, IN4 and IN5 refer to the inoculum size were 0.017 g/L, 0.060 g/L, 0.103 g/L, 0.135 g/L and 0.160 g/L, respectively.

Inoculum size also affected the nutrient removal ratio. Figure 4 shows that the similar dynamic profiles in removal ratio of TOC, TN and TP were observed. IN3 and IN4 runs achieved the high TOC removal ratio afterward day 8 and maximized at the end of test. As shown in Figure 4a, the removal ratios of TOC in IN3 and IN4 were 58.87% and 57.23%, respectively, both were higher than those in other treatments (p<0.05). At the end of the test, although there was no significant difference between IN3 and IN4, both reached the higher TN removal ratios with the value of 50%, compared to

that in other treatments (p<0.01). The highest TP removal ratio also occurred in IN3 (54.03%) and IN4 (57.35%), which were 15.7%-59.0% higher than those in the other treatments at the 6th day.



Note: The inoculum sizes of IN1, IN2, IN3, IN4 and IN5 was 0.017 g/L, 0.060 g/L, 0.103 g/L, 0.135 g/L and 0.160 g/L, respectively.

Figure 4 Removal ratio of nutrient at different inoculum sizes

In PHWW, phenols, phenolics and some organic nitrogen compounds (such as 2-piperidone, 2,2,6,6-tetramethyl-4-piperidone, 1-methyl-2pyrrolidinone) are toxic and have been shown to inhibit the growth of *Chlorella*[9,37]. Moreover, in the high concentration PHWW media, high ammonium may negatively affect microalgae growth, which all might prolonged the lag phase time. An appropriate inoculum size can help microalgae to rapidly build up a competitive advantage to adapt to the environment[37,38]. It was expected that the acclimation period can be expedited via appropriate inoculum size in high concentration of PHWW. Evidence exists that the dosage of algae in suspension is increased, the sensitivity of toxicity to algae cells will be alleviated[24,38]. In addition, a proper inoculum size could reduce the cell mortality ratio, and increase the biomass production and nutrient recovery. The cell mortality ratio may range from 20% to 80% subjected to stress, depending on strains, bioreactors, and stress conditions[39-41]. Wang et al. reported[41] that, with increase in initial biomass density from 0.1 g/L to 1.5 g/L, less cell mortality but greatly increased biomass accumulation were observed. In this work, the optimal inoculums size was from 0.103 g/L to 0.135 g/L, which was accordance with Want et al.'s report[41].

However, the physiologically activity was not increased as the inoculums size increased from IN1 to IN5. The μ_{max} of IN5 run was lower than those of IN4 and IN3. It was due in part to the influence of the inoculum size on light availability to individual microalgae cells. Higher inoculum size caused the higher frequency of light-dark cycle and the shorter light exposure time for individual microalgae cells. The higher value of μ_{max} in IN3 and IN4 was partially attributed to the desirable frequency of light-dark cycle and light exposure time. The strong mutual shading of cells in IN5 might cause the decrease of μ_{max}.

Although the results suggested an inoculums size of 0.103-0.135 g/L is most suitable for *C. vulgaris* 1067 to grow in small-scale laboratory cultures, in large-scale cultures, however, the inoculum size could be different and thus requires further investigation.

4 Conclusions

This study showed that *C. vulgaris* 1067 performed the best among the five screened microalgae strains. Biomass has reached 0.23 g/L in 4 d. In PHWW separated by ethyl ether, *C. vulgaris* 1067 could tolerate a concentration up to 7.6% PHWW media. The optimal PHWW concentration appeared 5.2% (TN500 run) with 0.84 g/L biomass production and 0.065 g/(L·d) daily productivity, while simultaneously maximized the nutrients removal quantity. By using the PHWW that pretreated by ethyl ether as the cultural medium, the initial tolerant concentration of microalgae was increased, which positively enhanced the biomass production and nutrients recovery quantity of *C. vulgaris* 1067. It is an important accomplishment for efficient realizing of the

E^2-Energy system. The desirable range of inoculum sizes were suggested between 0.103-0.135 g/L for *C. vulgaris* 1067 under laboratory conditions, which much shortens the retention time for nutrient recovery, thus improved biomass final daily productivity to 0.13 g/(L·d), 15.7-fold higher of that as reported in the literature. This research provides an important contribution to the advancement of utilizing wastewater from HTL processes to reuse spent nutrients and produce microalgae biomass for bioenergy production. As demonstrated in this research that as the microalgae used in this study are domesticated in PHWW media, the biomass productivity is largely increased based on the initial nutrients in fresh cultivation medium then proposed a promising technology for the microalgae-to-biofuels industry.

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