

Article

Effects of HTL Operating Conditions on Fuel-Related Properties and Compositional Evolution Microalgae-Derived Bio-Crude Oil

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Abstract

This study systematically investigated the hydrothermal liquefaction (HTL) of *Chlorella vulgaris* to understand how operating conditions influence bio-crude oil yield, nitrogen behavior, fuel properties, and compositional changes. A Box–Behnken design (BBD) was used, with temperature (200–300 °C), pressure (50–200 bar), and reaction time (15–45 min) as the process variables. Bio-crude oil yields ranged from 11.10 to 40.79 wt% (dry basis), and nitrogen content varied between 2.34 and 6.22 wt%. Temperature was the primary factor affecting both bio-crude oil yield and nitrogen content. Higher temperatures increased bio-crude oil production but also led to more nitrogen incorporating into the oil phase, indicating a trade-off between yield and nitrogen retention. Van Krevelen analysis showed that HTL bio-crude had lower O/C ratios than the raw microalgal feedstock, suggesting progressive deoxygenation under more severe HTL conditions. GC–MS analysis revealed that hydrocarbons increased and fatty acids decreased with rising temperature. Nitrogen-containing compounds such as amides and N-heterocyclic compounds, were still detectable at high temperatures. Multi-response optimization identified 300 °C, 200 bar, and 15 min as the optimal conditions, balancing maximized bio-crude oil yield with minimized nitrogen content. These results suggest that HTL bio-crude oil is regarded as an intermediate feedstock requiring further upgrading. Therefore, optimizing the HTL process should simultaneously consider both fuel-related properties and nitrogen behavior.

Keywords: hydrothermal liquefaction; *Chlorella vulgaris*; Box–Behnken design; response surface methodology; nitrogen behavior; higher heating value; GC–MS



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1. Introduction

Climate change has accelerated the global energy system's transition from conventional fossil fuel-based systems to those reliant on renewable energy [1]. Within this transition,

biofuels derived from biomass have garnered increasing attention as sustainable energy alternatives [2]. Liquid biofuels, in particular, are attractive due to their compatibility with existing energy infrastructure [3]. These fuels remain essential for transportation and industrial sectors because of their high energy density and ease of storage and transport [3]. Consequently, the development of renewable liquid fuels has attracted growing interest, and biomass is widely considered a promising feedstock for their production [2,4].

Among various biomass resources, microalgae have received considerable attention as a promising feedstock for biofuel production due to their rapid growth rate, high productivity, ability to be cultivated on non-arable land, and minimal competition with food resources [5]. *Chlorella vulgaris* has been extensively studied as one of the commercially relevant microalgae for biofuel production owing to its relatively high lipid content, robust growth characteristics, and well-documented biochemical composition. Previous studies report that the lipid content of *C. vulgaris* generally ranges from approximately 15–70 wt% on a dry weight basis and can be further enhanced under mixotrophic cultivation conditions [6]. Additionally, *C. vulgaris* has been shown to maintain high growth rates ($\mu_{\max} \approx 0.6\text{--}0.95$) and biomass productivity exceeding 800 mg L^{-1} even under suboptimal temperature conditions (approximately $18\text{--}19^\circ\text{C}$), highlighting its adaptability and suitability for large-scale cultivation [7]. These characteristics make *C. vulgaris* an appropriate model feedstock for evaluating HTL performance and the effects of operating conditions on bio-crude oil production and fuel-related properties.

However, the high moisture content of microalgae significantly limits conventional thermochemical conversion processes, which typically require energy-intensive drying before fuel production. To overcome this limitation, hydrothermal liquefaction (HTL) has emerged as a promising thermochemical conversion technology for wet biomass [8].

HTL is a thermochemical process conducted under subcritical water conditions, typically at temperatures of $250\text{--}374^\circ\text{C}$ and pressures of $10\text{--}25 \text{ MPa}$ [8]. Under these conditions, water exhibits a decreased dielectric constant, reduced viscosity, and an increased ionic product (K_w), resulting in lower polarity and solvent properties comparable to those of organic solvents [8,9]. Consequently, the solubility of various organic compounds is enhanced, and water acts as a highly reactive reaction medium [9]. During HTL, major biomass components such as lipids, proteins, and carbohydrates undergo thermochemical reactions including hydrolysis, dehydration, decarboxylation, deoxygenation, and cracking, ultimately leading to the formation of energy-dense bio-crude oil [10]. The extent of these reactions is strongly governed by HTL reaction severity, which directly influences product distribution and fuel characteristics [11].

Compared to other thermochemical conversion processes, HTL offers several advantages, including the direct processing of high-moisture biomass without a drying step, thereby significantly reducing energy consumption associated with water evaporation [12]. HTL has also been reported to achieve high energy recovery efficiencies of approximately 85–90% and carbon recoveries exceeding 70%, indicating high efficiency in both energy and resource utilization [12]. Furthermore, HTL-derived bio-crude oil generally exhibits higher carbon content, lower oxygen content, and elevated higher heating values (HHVs) of approximately $30\text{--}50 \text{ MJ/kg}$ compared to raw biomass. This energy density exceeds that of pyrolysis oil ($24\text{--}34 \text{ MJ/kg}$) and approaches that of petroleum-derived crude oil ($\sim 41 \text{ MJ/kg}$), demonstrating its potential as a renewable liquid fuel intermediate [12,13].

Despite these advantages, the physicochemical properties and fuel characteristics of HTL-derived bio-crude oil remain highly dependent on operating conditions such as temperature, pressure, and reaction time [14,15]. Recent HTL studies have expanded their scope to include catalytic HTL, continuous-flow operation, and industrial-scale implementation [16,17]. In catalytic HTL, key operating variables and catalyst selection influence

bio-crude oil formation and quality-improving reactions [16], while continuous systems necessitate well-defined operating windows for stable conversion and product recovery [17]. Therefore, a clear understanding of how operating conditions affect bio-crude oil yield, heteroatom content, and fuel-related properties is essential for producing upgradeable microalgae-derived bio-crude oil [15,18].

Although numerous studies have investigated microalgae HTL, most have primarily focused on maximizing bio-crude oil yield or evaluating selected fuel properties under specific operating conditions [19]. Relatively limited attention has been given to the simultaneous assessment of bio-crude oil yield, nitrogen retention, fuel-related properties, and compositional evolution [15,18–20]. In particular, the trade-off between bio-crude oil production and nitrogen retention remains insufficiently understood, despite its importance for downstream upgrading. Accordingly, a systematic evaluation of HTL operating conditions that considers both bio-crude oil quantity and quality is still needed.

In this study, HTL of *C. vulgaris* was systematically investigated using a Box–Behnken design (BBD). *C. vulgaris* was selected as a suitable feedstock due to its relatively high protein content, allowing for the evaluation both bio-crude oil yield and nitrogen content under different HTL operating conditions. Temperature (200–300 °C), pressure (50–200 bar), and reaction time (15–45 min) were chosen as key process variables. Bio-crude oil yield and nitrogen content were selected as the primary response variables for optimization, representing production efficiency and a key upgrading-related constraint of microalgae-derived HTL bio-crude oil, respectively. To further interpret the fuel-intermediate quality of the produced bio-crude oils, H/C ratio, O/C ratio, HHV, ER, and GC–MS-based compositional characteristics were evaluated as complementary fuel-related indicators. Elemental and Van Krevelen analyses were used to assess changes in H/C and O/C ratios, HHV and ER were used to examine energy-related performance, and GC–MS analysis was conducted to interpret compound-level compositional changes under different HTL operating conditions. By integrating BBD-based process optimization with elemental, energy-based, and compositional evaluations, this study provides a systematic assessment of HTL operating conditions and applies multi-response optimization to identify a compromise condition that balances bio-crude oil quantity and quality, producing microalgae-derived bio-crude oil as an upgrading-oriented intermediate feedstock.

2. Materials and Methods

2.1. Microalgal Biomass

The microalgal biomass (*C. vulgaris*) used in this study was supplied by CNS INT (Hwaseong, Republic of Korea) as a wet slurry. The slurry had a total solids (TS) content of 20 wt%, corresponding to a moisture content of approximately 80 wt%. The biochemical composition, elemental composition, and HHV of the microalgal biomass are presented in Table 1.

Table 1. Composition of *C. vulgaris*.

Biological Composition		Elemental Composition		Fuel-Related Property	
Carbohydrates (wt%)	33.58	C (wt%)	48.42	HHV (kcal/kg)	5343.12
Proteins (wt%)	47.60	H (wt%)	7.60		
Lipids (wt%)	13.44	N (wt%)	8.00		
Ashes (wt%)	5.39	S (wt%)	0.10		
		O ^a (wt%)	35.88		

^a By difference.

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filter paper to separate the solid residue from the liquid fraction. The liquid fraction was subsequently transferred to a separatory funnel to separate the organic (DCM-soluble) phase from the aqueous phase [22]. After phase separation, the DCM-rich organic phase was collected for bio-crude oil recovery. The solvent was then removed by natural evaporation. After most of the solvent had visibly evaporated, the sample was weighed at 2 h intervals, and complete solvent removal was defined as the point at which no further change in mass was observed. This constant-weight criterion was used to verify solvent removal before yield calculation. Possible material losses during sample transfer, filtration, phase separation, and solvent evaporation could not be completely excluded; therefore, the same extraction, separation, and solvent removal procedures were applied to all experimental runs to minimize procedural bias. The recovered bio-crude oil was used for yield determination, and the bio-crude oil yield was calculated based on the initial dry mass of microalgal biomass loaded into the reactor, as shown in Equation (1) [23].

$$\text{Bio-crude oil yield (wt\%, dry basis)} = \frac{W_{BC}}{W_{slurry} \times TS} \times 100 \quad (1)$$

where W_{BC} is the mass (g) of the recovered bio-crude oil, W_{slurry} is the mass (g) of the microalgal slurry loaded into the reactor, and TS is the total solids content of the slurry expressed as a mass fraction.

Energy recovery (ER) was calculated to evaluate the fraction of feedstock energy recovered in the bio-crude oil phase, as shown in Equation (2).

$$\text{ER(\%)} = \frac{Y_{BC} \times \text{HHV}_{BC}}{\text{HHV}_{feedstock}} \quad (2)$$

where Y_{BC} is the bio-crude oil yield (wt%, dry basis), HHV_{BC} is the higher heating value of the recovered bio-crude oil, and $\text{HHV}_{feedstock}$ is the higher heating value of the raw microalgal feedstock. The same energy unit was used for HHV_{BC} and $\text{HHV}_{feedstock}$ in the ER calculation. Because Y_{BC} is expressed in wt%, Equation (2) directly provides ER as a percentage.

2.3. Analytical Methods

The elemental composition of the bio-crude oil samples, including carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) contents, was determined for each experimental condition using a FlashSmart elemental analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Ash content was measured separately by ashing in a muffle furnace. Oxygen (O) content was calculated by difference $100\% - C - H - N - S - \text{ash}$. The higher heating value (HHV) of all bio-crude oil samples was measured using a LECO AC600 isoperibol calorimeter (LECO Corporation, St. Joseph, MI, USA).

Chemical profiles and major compound distributions of bio-crude oil samples from all 15 experimental runs were analyzed by GC–MS. Before analysis, each sample was diluted in dichloromethane (DCM) and filtered through a 0.45 μm nylon membrane filter. The GC–MS system comprised of an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled with an Agilent 5973 Inert mass selective detector (Agilent Technologies, Santa Clara, CA, USA), and compound separation was achieved using an HP–5MS capillary column (Agilent Technologies, Santa Clara, CA, USA; 30 m $\times$ 0.25 mm i.d., 0.25 μm film thickness). Detected compounds were tentatively identified by matching their mass spectra with the Wiley mass spectral library using MSD ChemStation software (version E.02.02; Agilent Technologies, Santa Clara, CA, USA). Only compounds with a library match similarity of 90% or higher were included in the compositional interpretation. Since calibration with authentic standards was not performed, the GC–MS results were

treated as semi-quantitative data and presented as normalized relative peak areas (%). The relative distribution of compound groups was calculated based on peak area normalization, allowing for discussion rather than absolute quantification.

2.4. Design of Experiments

In this study, a three-level Box–Behnken design (BBD) was applied using temperature (X_1), pressure (X_2), and reaction time (X_3) as independent variables to optimize bio-crude oil yield and investigate compositional variations in the bio-crude oil under different operating conditions [24]. The coded and actual levels of the independent variables are presented in Table 2. The response variables were defined as bio-crude oil yield and fuel-related properties of the bio-crude oil.

Table 2. Independent variables and coded levels used in the Box–Behnken design.

Variable	Symbol	Variable Levels		
		Low (−1)	Center (0)	High (+1)
Temperature (°C)	X_1	200	250	300
Pressure (bar)	X_2	50	125	200
Reaction Time (min)	X_3	15	30	45

Factor ranges were set at 200–300 °C for temperature, 50–200 bar for pressure, and 15–45 min for reaction time. These ranges were selected based on previous studies to cover technically relevant conditions for the hydrothermal liquefaction process. According to the three-factor Box–Behnken design (BBD) 12 non-center design points were generated and conducted as single experimental runs. The center-point condition (250 °C, 125 bar, and 30 min) was replicated three times to estimate experimental pure error and evaluate lack-of-fit and model adequacy. Therefore, the final experimental design consisted of 15 runs. Because only the center-point condition was replicated, standard deviations were calculated for the center-point responses only, while the non-center design points are reported as individual experimental values. Experimental design generation and statistical analyses were performed using Minitab 14 software (Minitab Inc., State College, PA, USA). The run order was randomized to minimize systematic error. The randomized run order and coded factor combinations for each run are presented in Table 3.

Table 3. Randomized run order and coded factor combinations in the Box–Behnken design.

Run Order	Coded Factor		
	X_1 (Temperature, °C)	X_2 (Pressure, Bar)	X_3 (Reaction Time, Min)
1	300 (+1)	125 (0)	45 (+1)
2	250 (0)	125 (0)	30 (0)
3	250 (0)	125 (0)	30 (0)
4	250 (0)	50 (−1)	15 (−1)
5	250 (0)	200 (+1)	45 (+1)
6	300 (+1)	50 (−1)	30 (0)
7	200 (−1)	200 (+1)	30 (0)
8	200 (−1)	125 (0)	45 (+1)
9	300 (+1)	200 (+1)	30 (0)
10	200 (−1)	50 (−1)	30 (0)
11	250 (0)	125 (0)	30 (0)
12	200 (−1)	125 (0)	15 (−1)
13	250 (0)	50 (−1)	45 (+1)
14	250 (0)	200 (+1)	15 (−1)
15	300 (+1)	125 (0)	15 (−1)

Regression analysis for each response variable was performed using a second-order polynomial model including linear, quadratic, and interaction terms, and the general regression equation is given in Equation (3).

$$Y = \beta_0 + \sum_{i=1}^3 \beta_i x_i + \sum_{i=1}^3 \beta_{ii} x_i^2 + \sum_{i < j} \beta_{ij} x_i x_j \quad (3)$$

where Y is the predicted response, β_0 is the intercept, β_i , β_{ii} , and β_{ij} are the regression coefficients for the linear, quadratic, and interaction terms, respectively, and x_i and x_j are the dimensionless coded values of the independent variables. The statistical significance of each term was evaluated by analysis of variance (ANOVA), and a p -value of less than 0.05 was considered statistically significant. These operating ranges were selected to represent varying HTL reaction severities ranging from relatively mild to severe thermochemical conversion conditions.

3. Results

3.1. HTL Experimental Results

The bio-crude oil yield and nitrogen content obtained under each Box–Behnken design (BBD) condition are presented in Table 4.

Table 4. Experimental bio-crude oil yield and nitrogen content obtained under the Box–Behnken design conditions.

Run	Temperature °C	Pressure Bar	Reaction Time Min	Bio-Crude Oil Yield wt%, Dry Basis	Nitrogen Content wt%
1	300 (+1)	125 (0)	45 (+1)	34.83	5.92
2	250 (0)	125 (0)	30 (0)	26.65	6.22
3	250 (0)	125 (0)	30 (0)	32.97	5.80
4	250 (0)	50 (−1)	15 (−1)	24.55	5.71
5	250 (0)	200 (+1)	45 (+1)	34.11	5.82
6	300 (+1)	50 (−1)	30 (0)	32.84	5.34
7	200 (−1)	200 (+1)	30 (0)	19.92	2.34
8	200 (−1)	125 (0)	45 (+1)	19.23	3.34
9	300 (+1)	200 (+1)	30 (0)	32.32	5.35
10	200 (−1)	50 (−1)	30 (0)	11.10	3.21
11	250 (0)	125 (0)	30 (0)	25.71	5.60
12	200 (−1)	125 (0)	15 (−1)	17.12	2.90
13	250 (0)	50 (−1)	45 (+1)	29.60	5.56
14	250 (0)	200 (+1)	15 (−1)	34.10	5.25
15	300 (+1)	125 (0)	15 (−1)	40.79	5.41

The individual values from all 15 experimental runs were retained in Table 4 for fitting the BBD-based regression models. Only the center-point condition was replicated. For this replicated center-point condition, the average bio-crude oil yield and nitrogen content were 28.44 ± 3.95 wt% and 5.87 ± 0.32 wt%, respectively. These replicated data were used to estimate experimental pure error and to evaluate lack-of-fit and model adequacy through ANOVA.

Bio-crude oil yield ranged from 11.10 to 40.79 wt% (dry basis), which is consistent with values commonly reported for microalgal HTL studies [25]. Specifically, yields obtained at 250–300 °C, particularly the maximum yield of 40.79 wt%, were comparable to those reported in a recent high-solid-content HTL study using *C. vulgaris*. Wang et al. [26] reported bio-oil yields of 23.83–48.48 wt% and nitrogen contents of 7.76–10.19 wt% across their investigated experimental conditions. In comparison, bio-crude oils produced at 300 °C in

the present study showed comparable yields (32.32–40.79 wt%) but lower nitrogen contents (5.34–5.92 wt%). This comparison suggests that high-yield HTL conditions for *C. vulgaris* can still be associated with substantial nitrogen retention, highlighting the need to evaluate bio-crude oil yield alongside nitrogen content. At 200 °C, relatively lower nitrogen contents of 2.34–3.34 wt% were observed, but the corresponding bio-crude oil yields were also low, ranging from 11.10 to 19.92 wt%. This trend aligns with previous reports indicating that mild HTL conditions can limit nitrogen retention in the bio-crude oil phase, whereas higher-severity conditions can increase both bio-crude oil formation and nitrogen retention. For example, the nitrogen content of *Chlorella* bio-crude oil was reported to increase from 2.6 wt% at 250 °C to 7.5 wt% at 300 °C under continuous HTL conditions [27]. Additionally, HTL bio-oil is commonly reported to contain approximately 3–6 wt% nitrogen, and high bio-oil yield can be accompanied by high nitrogen content [28]. Therefore, the present results suggest a trade-off between bio-crude oil yield and nitrogen retention, supporting the need to evaluate HTL operating conditions based on both responses rather than yield alone.

3.2. Regression Model Fitting and Adequacy Assessment

3.2.1. ANOVA Results and Model Adequacy of the Fitted Quadratic Models

To quantitatively interpret the relationships between process variables and response variables, quadratic regression models based on the Box–Behnken design (BBD) were developed for bio-crude oil yield and nitrogen content. The statistical significance and adequacy of the fitted models were evaluated using analysis of variance (ANOVA), lack-of-fit tests, and coefficients of determination (R^2). The ANOVA results and model adequacy parameters for the fitted quadratic regression models are summarized in Table 5.

Table 5. Summary of ANOVA results and adequacy statistics for the fitted quadratic models.

Response	Model <i>p</i> -Value	Linear <i>p</i> -Value	Quadratic <i>p</i> -Value	Interaction <i>p</i> -Value	Lack-of-Fit <i>p</i> -Value	R^2 (%)	Adjusted R^2 (%)	Significant Terms
Bio-crude oil yield	0.010	0.104	0.201	0.605	0.786	94.7	85.2	X_1
Nitrogen content	0.000	0.000	0.001	0.186	0.828	98.7	96.4	X_1, X_1^2

The fitted regression models for both bio-crude oil yield and nitrogen content were statistically significant. Additionally, the insignificant lack-of-fit results for both models indicated satisfactory model adequacy within the investigated experimental range. The coefficients of determination (R^2) were 94.7% and 98.7% for bio-crude oil yield and nitrogen content, respectively, demonstrating that the developed quadratic models adequately described the variations in the two response variables.

Notably, the nitrogen-content model exhibited higher explanatory power than the bio-crude oil yield model, suggesting that nitrogen behavior responded more consistently to variations in HTL operating conditions. In contrast, the effects of interaction terms were relatively limited for both responses. Based on the ANOVA results, temperature was identified as the statistically dominant operating parameter within the investigated experimental range, whereas the influences of pressure and reaction time were comparatively less pronounced. Considering that pressure in HTL is closely associated with the maintenance of a compressed liquid-water environment, the pressure term in this study was interpreted as an imposed operating condition rather than as a primary independent reaction-driving factor.

3.2.2. Significance of Regression Coefficients in the Fitted Quadratic Models

The regression coefficients and statistical significance of each model term are presented in Table 6.

Table 6. Regression coefficients and *p*-values of the fitted quadratic models.

Term	Bio-Crude Oil Yield Coefficient	Bio-Crude Oil Yield <i>p</i> -Value	Nitrogen Content Coefficient	Nitrogen Content <i>p</i> -Value
Constant	−143.993	0.027	−36.3806	0.000
X_1	1.042	0.027	0.3181	0.000
X_2	0.267	0.103	−0.0074	0.505
X_3	0.010	0.989	−0.0203	0.723
X_1^2	−0.001	0.083	−0.0006	0.000
X_2^2	−0.000	0.603	−0.0001	0.056
X_3^2	0.014	0.119	0.0001	0.867
$X_1 X_2$	−0.001	0.194	0.0001	0.127
$X_1 X_3$	−0.003	0.252	0.0000	0.890
$X_2 X_3$	−0.001	0.455	0.0002	0.195

According to the significance analysis of the regression terms in Table 6, temperature (X_1) was the only statistically significant variable in the bio-crude oil yield model ($p = 0.027$). Pressure (X_2), reaction time (X_3), quadratic terms, and interaction terms were not statistically significant. These results indicate that temperature was the primary factor influencing bio-crude oil yield within the experimental range investigated. This observation aligns with previous studies reporting temperature as the dominant process variable affecting bio-crude oil yield and product characteristics during microalgal HTL [29].

In contrast, both the linear (X_1) and quadratic (X_1^2) terms for temperature were statistically significant in the nitrogen-content model ($p < 0.001$), indicating a nonlinear response of nitrogen content to HTL temperature. The linear terms for pressure and reaction time, along with the interaction terms, were generally insignificant, while the quadratic term for pressure (X_2^2) showed marginal significance ($p = 0.056$). Overall, temperature was the dominant factor affecting both response variables, with pressure and reaction time having comparatively limited influence. The limited effect of pressure suggests that, within the investigated range, it primarily served to maintain the hydrothermal reaction environment rather than independently governing bio-crude oil yield or nitrogen content. These findings are also consistent with previous studies reporting that only a fraction of feedstock nitrogen is transferred into the bio-oil phase during HTL, potentially leading to a trade-off between bio-crude oil yield and nitrogen retention [28,30].

The predictive equations for bio-crude oil yield and nitrogen content, based on the fitted quadratic regression models, are presented in Equations (4) and (5), respectively.

$$Y_{\text{yield}} = -143.993 + 1.042X_1 + 0.267X_2 + 0.010X_3 - 0.001X_1^2 + 0.014X_3^2 - 0.001X_1X_2 - 0.003X_1X_3 - 0.001X_2X_3 \quad (4)$$

$$Y_N = -36.3806 + 0.3181X_1 - 0.0074X_2 - 0.0203X_3 - 0.006X_1^2 - 0.001X_2^2 + 0.001X_3^2 + 0.001X_1X_2 + 0.002X_2X_3 \quad (5)$$

Here, Y_{yield} represents bio-crude oil yield (wt%, dry basis), and Y_N represents nitrogen content in bio-crude oil (wt%). The variables X_1 , X_2 , and X_3 denote the actual values of temperature (°C), pressure (bar), and reaction time (min), respectively.

3.2.3. Regression Validation Using Residual and Predicted-Versus-Observed Plots

The regression validation plots for the fitted quadratic models of bio-crude oil yield and nitrogen content are presented in Figure 2.

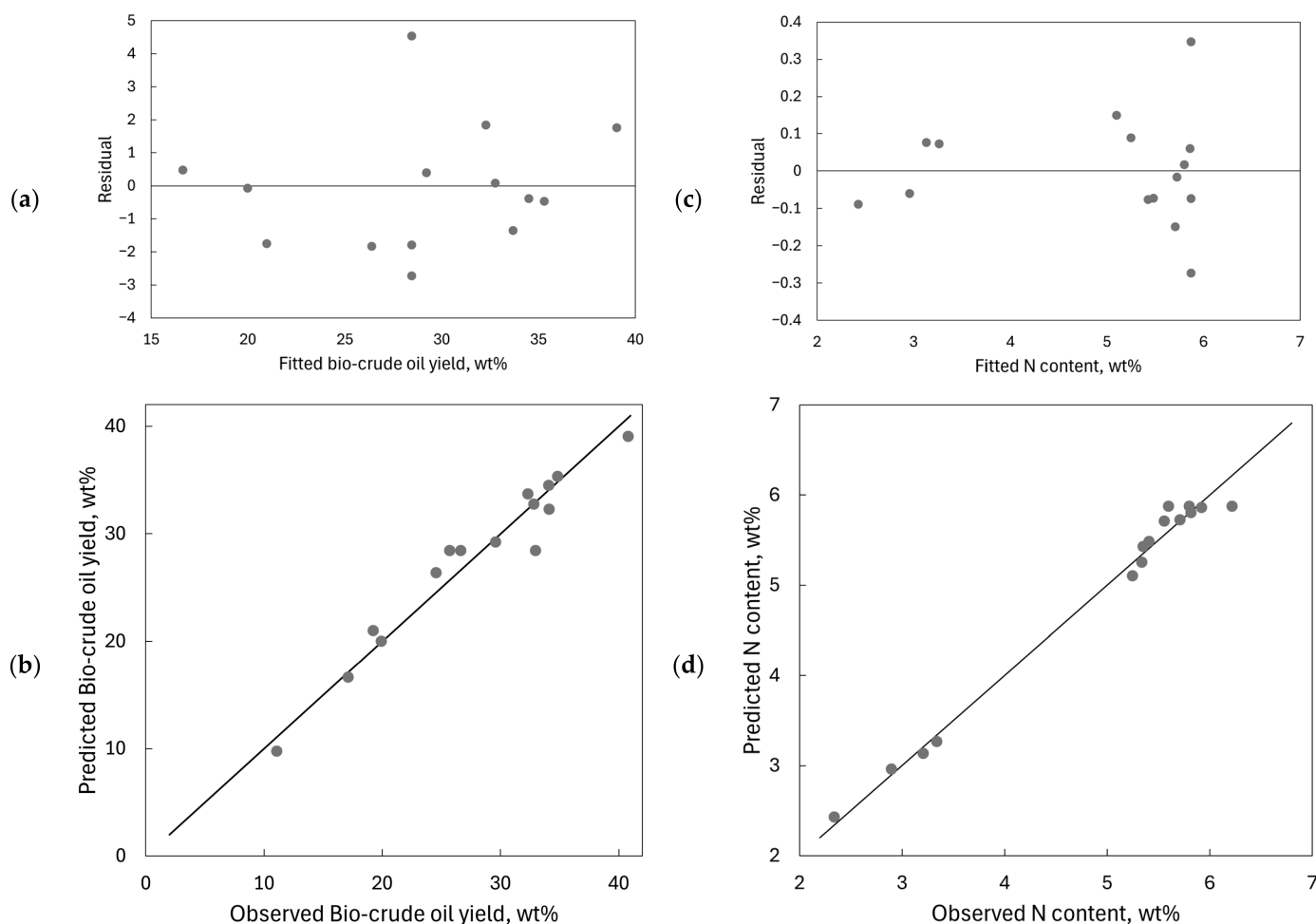


Figure 2. Regression validation plots for the fitted quadratic models: (a) residuals versus fitted values for bio-crude oil yield, (b) residuals versus fitted values for nitrogen content, (c) predicted versus observed plot for bio-crude oil yield, and (d) predicted versus observed plot for nitrogen content.

The predicted versus observed plots showed that the model-predicted values were generally distributed close to the 1:1 reference line for both responses, indicating reasonable agreement between the experimental and predicted values. In addition, the residuals were distributed around zero without a clear systematic trend in the residuals versus fitted values plots. The absolute residuals were within ± 5 in the corresponding response units, indicating that the differences between the experimental and model-predicted values were limited within the investigated BBD range. These results support the acceptable adequacy of the fitted quadratic models for interpreting the response trends of bio-crude oil yield and nitrogen content.

3.3. Effects of Process Variables on Bio-Crude Oil Yield and Nitrogen Content

The individual effects of temperature, pressure, and reaction time predicted from the fitted quadratic regression models are presented in Figures 3 and 4.

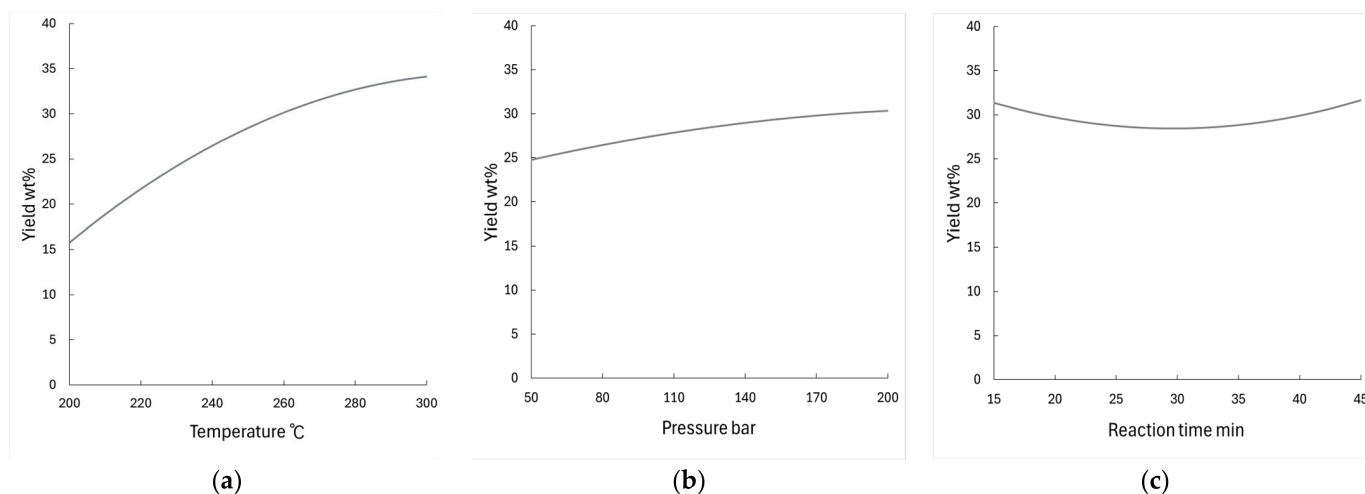


Figure 3. Effects of (a) temperature, (b) pressure, and (c) reaction time on bio-crude oil yield.

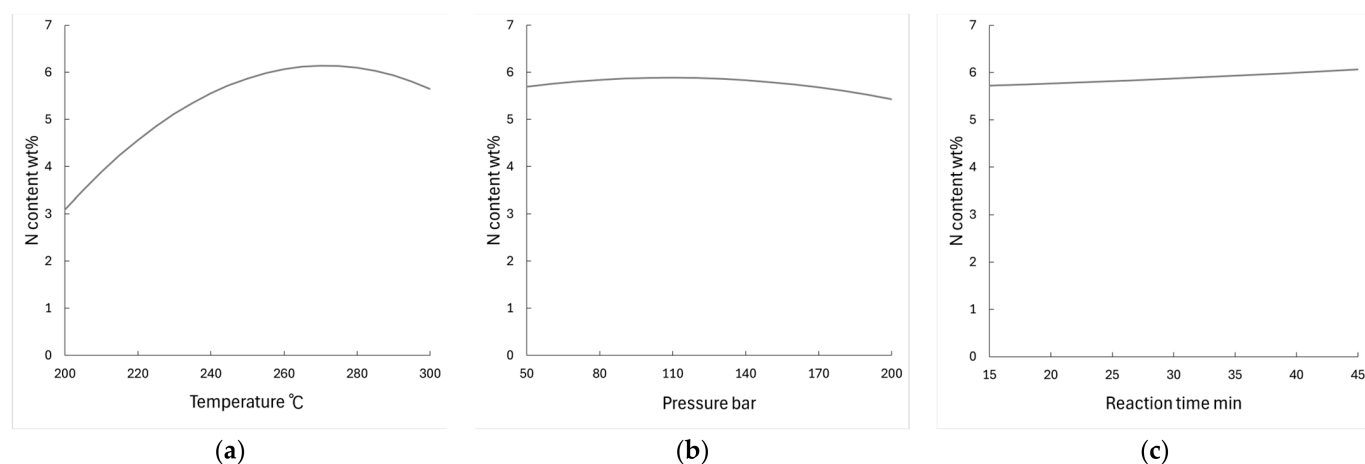


Figure 4. Effects of (a) temperature, (b) pressure, and (c) reaction time on nitrogen content in bio-crude oil.

In each plot, only one variable was varied, while the other two were fixed at their center-point values (temperature: 250 °C, pressure: 125 bar, and reaction time: 30 min). Overall, temperature had the strongest influence on both bio-crude oil yield and nitrogen content, with pressure and reaction time showing comparatively moderate effects.

Bio-crude oil yield increased markedly with increasing temperature (Figure 3a). In contrast, the predicted change in bio-crude oil yield along the pressure axis was relatively gradual (Figure 3b), supporting the ANOVA result that pressure had a limited independent effect within the investigated range. Reaction time exhibited a weak U-shaped trend, with slightly lower yields near 30 min; however, the overall variation remained limited (Figure 3c). These results indicate that the most pronounced variation in bio-crude oil yield occurred along the temperature axis, with higher temperatures favoring bio-crude oil production under the investigated HTL conditions. This observation is consistent with previous studies reporting that temperature is the primary process variable governing bio-crude oil yield during microalgal HTL [31].

Nitrogen content in the bio-crude oil exhibited a nonlinear response to temperature (Figure 4a). The lowest nitrogen contents were observed near 200 °C, followed by an increase with rising temperature, reaching a maximum near 270 °C, and then showing a slight decrease at higher temperatures. This behavior suggests that the decomposition, transformation, and phase partitioning of protein-derived nitrogen compounds during

microalgal HTL are highly temperature-dependent [32]. Mechanistically, under relatively mild HTL conditions, protein-derived nitrogen may be released as water-soluble amino acids through hydrolysis and further converted into ammonia and amine-containing intermediates through deamination, thereby limiting nitrogen retention in the bio-crude oil phase [32,33]. As temperature increases, these nitrogen-containing intermediates can participate in secondary reactions with lipid- and carbohydrate-derived intermediates, including condensation and Maillard-type reactions, forming amides and N-heterocyclic compounds that can be retained in the bio-crude oil phase [34–36]. Therefore, the nonlinear nitrogen response observed in this study can be interpreted as the combined result of nitrogen transformation, retention in the bio-crude oil phase, and redistribution into non-oil phases [18,33–36]. The influence of pressure on nitrogen content was relatively moderate (Figure 4b), whereas reaction time showed a slight increasing trend with increasing reaction duration (Figure 4c). However, the overall effects of pressure and reaction time remained considerably smaller than those associated with temperature.

Taken together, the predicted trends for bio-crude oil yield and nitrogen content indicate that increasing temperature favors bio-crude oil production but may simultaneously increase nitrogen retention in the oil phase, resulting in a trade-off relationship between the two responses. This tendency is consistent with previous studies reporting a compromise between bio-oil yield and nitrogen retention during HTL processing [37]. Specifically, low-temperature conditions were advantageous for nitrogen reduction but unfavorable for bio-crude oil production, whereas high-temperature conditions promoted bio-crude oil yield while increasing the possibility of nitrogen incorporation into the bio-crude oil phase. These findings support the need for subsequent multi-response optimization to identify balanced HTL operating conditions.

3.4. Multi-Response Compromise Optimization of Bio-Crude Oil Production

To determine compromise optimum conditions that simultaneously maximize bio-crude oil yield and minimize nitrogen content in the bio-crude oil, multi-response optimization was performed based on the fitted quadratic regression models. The optimization results are presented in Figure 5.

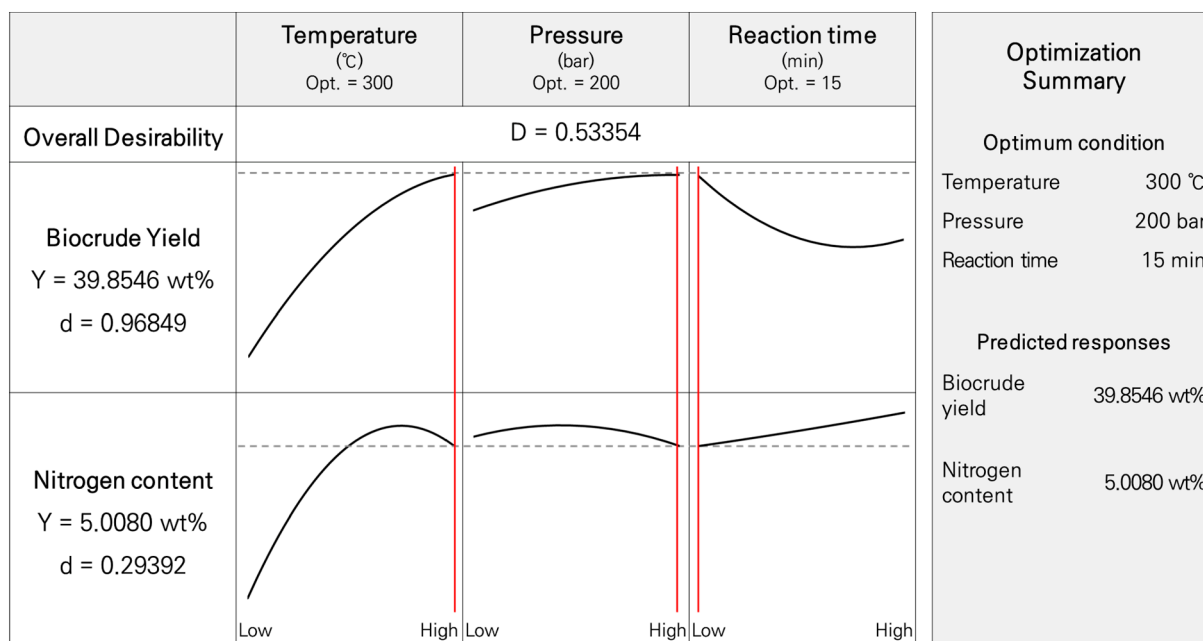


Figure 5. Multi-response compromise optimization plot for bio-crude oil production based on bio-crude oil yield and nitrogen content.

According to the multi-response optimization results shown in Figure 5, the compromise optimum condition, considering both maximum bio-crude oil yield and minimum nitrogen content, was determined to be 300 °C, 200 bar, and 15 min. Under these conditions, the predicted bio-crude oil yield and nitrogen content were 39.8546 wt% and 5.00796 wt%, respectively. The overall desirability value (D) was calculated as 0.53354, while the individual desirability values (d) for bio-crude oil yield and nitrogen content were 0.96849 and 0.29392, respectively.

The moderate overall desirability was primarily due to the low individual desirability for nitrogen minimization, despite the high individual desirability for bio-crude oil yield. This indicates that maximizing bio-crude oil yield and minimizing nitrogen retention in the bio-crude oil phase were partially conflicting objectives within the investigated HTL range. Low-temperature conditions reduced nitrogen retention but resulted in low bio-crude oil yields, whereas high-temperature conditions enhanced bio-crude oil production but increased nitrogen retention. Therefore, the derived optimum condition should be interpreted as a practical compromise for producing bio-crude oil as an intermediate feedstock requiring downstream upgrading, rather than a condition that simultaneously achieves ideal yield maximization and nitrogen minimization [28]. In fact, microalgal HTL bio-crude oil generally requires further upgrading due to the presence of heteroatoms such as nitrogen and oxygen, while overall process feasibility is strongly influenced by bio-crude oil yield and production efficiency [38].

Therefore, the present study focused on identifying a balanced optimum condition capable of maintaining nitrogen content within an upgradeable range while simultaneously achieving a practically meaningful bio-crude oil yield, rather than targeting the absolute minimum nitrogen content. These results also reflect the trade-off relationship identified in the individual factor analysis, where increasing temperature promoted bio-crude oil production but could simultaneously increase nitrogen retention in the oil phase. Furthermore, the derivation of the upper temperature limit (300 °C) as the optimum condition further confirms that temperature was the dominant factor contributing to bio-crude oil yield enhancement within the investigated HTL range. Because the aqueous, gaseous, and solid products were not quantitatively characterized for all BBD runs, the derived condition should be interpreted as an oil-phase compromise based on bio-crude oil yield and nitrogen content, rather than as a complete process-efficiency optimum based on phase-wise product distribution or carbon recovery.

3.5. Evaluation of HHV–Nitrogen Characteristics and Energy Recovery of Bio-Crude Oils

3.5.1. Van Krevelen Analysis for Fuel Precursor Characteristics of Bio-Crude Oil

Figure 6 presents the Van Krevelen diagram comparing the raw microalgal feedstock and the bio-crude oil samples obtained under different HTL conditions with literature-reported regions of petroleum crude oil, non-catalytic pyrolysis oil, and hydrotreated bio-crude oil.

All bio-crude oil samples exhibited significantly lower O/C ratio compared to the raw microalgal feedstock, indicating deoxygenation during the HTL process and improved elemental characteristics or fuel applicability. Specifically, the raw *C. vulgaris* feedstock had O/C and H/C ratios of approximately 0.56 and 1.87, respectively, while the HTL bio-crude oil samples showed much lower O/C ratios (0.11–0.27) and H/C ratios (1.49–1.72). This shift suggests that oxygen-containing functional groups in the raw microalgal biomass were partly removed during HTL. Concurrently, the decrease in the H/C ratio indicates that dehydration, decarboxylation, and condensation reactions may have occurred under hydrothermal conditions. This trend aligns with recent review studies reporting that

HTL promotes oxygen removal during the conversion of wet biomass into energy-dense bio-crude oil [39].

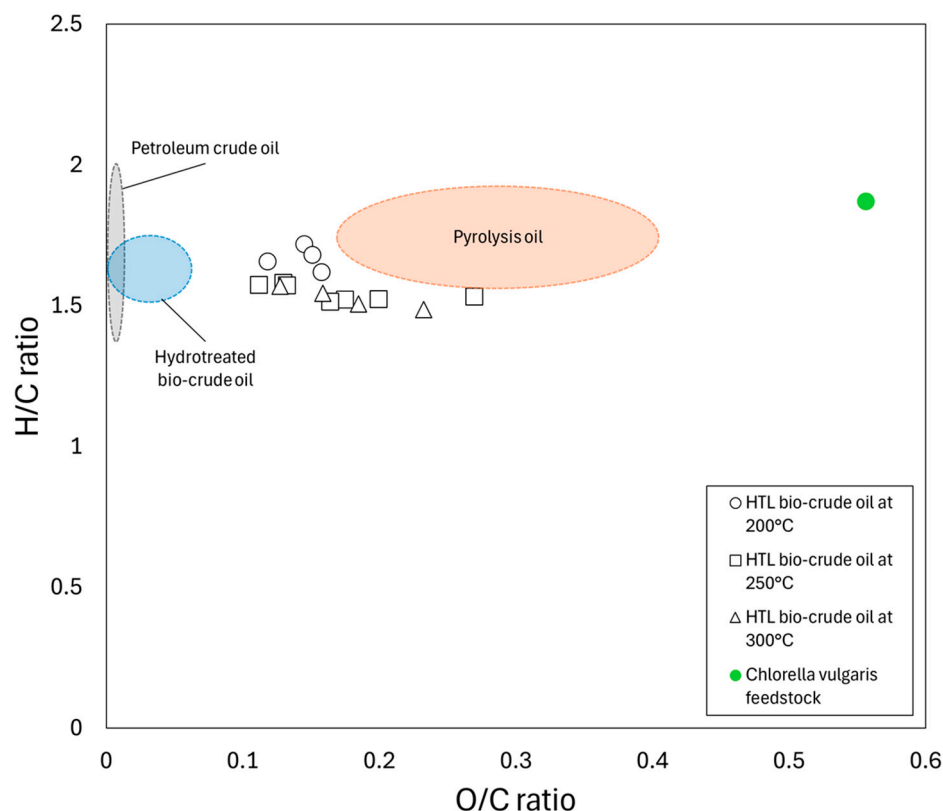


Figure 6. Van Krevelen diagram of the raw *C. vulgaris* feedstock and HTL bio-crude oils obtained under different operating conditions, with literature-reported petroleum crude oil, non-catalytic pyrolysis oil, and hydrotreated bio-crude oil regions as references.

Regarding the distribution under different temperature conditions, bio-crude oil samples obtained at 300 °C generally tended to have lower H/C ratios, and some also exhibited relatively lower O/C ratios compared to those produced at 200 °C and 250 °C. These results suggest that more severe HTL conditions promoted deoxygenation reactions, accompanied by dehydration, decarboxylation, and condensation reactions, thereby altering in the elemental characteristics of the bio-crude oil [40,41]. When compared with the literature-reported non-catalytic pyrolysis oil region (approximately O/C = 0.17–0.40 and H/C = 1.60–1.90 [42]), the HTL bio-crude oil samples were positioned near the lower-O/C side of this region, with partial overlap in O/C ratios. However, their H/C ratios were generally lower than those of the pyrolysis oil region. Additionally, partial overlap among samples obtained at 200–300 °C was observed, particularly in the O/C ratio distribution. Although decreasing O/C ratios were generally associated with increasing temperature, the distributions under different conditions were not completely separated. Therefore, increasing temperature within the investigated HTL range generally shifted the elemental characteristics of the bio-crude oils toward lower O/C ratios, despite some overlap among conditions.

Furthermore, the compromise optimum condition derived in Section 3.4 was located in the high-temperature region (300 °C), indicating that this condition was favorable not only in terms of bio-crude oil yield but also for elemental fuel characteristics. Nevertheless, all bio-crude oil samples remained clearly distinguishable from the petroleum crude oil region, which is characterized by very low O/C ratios and H/C ratios in the hydrocarbon-rich fuel range [43]. In contrast, the hydrotreated bio-crude oil region shown in Figure 6

was positioned closer to the petroleum crude region, with substantially lower O/C ratios (approximately 0.02–0.06) and H/C ratios closer to those of petroleum crude [44]. This comparison suggests that the bio-crude oils produced in this study should be regarded as fuel intermediates with potential for further improvement through downstream upgrading, particularly hydrotreating, rather than a final transportation fuel [40,41].

3.5.2. Evaluation of Nitrogen Content and Higher Heating Value of Bio-Crude Oil

Figure 7 compares the nitrogen content and higher heating value (HHV) distributions of the bio-crude oil samples obtained under different HTL conditions with those of petroleum crude oil and the raw microalgal feedstock.

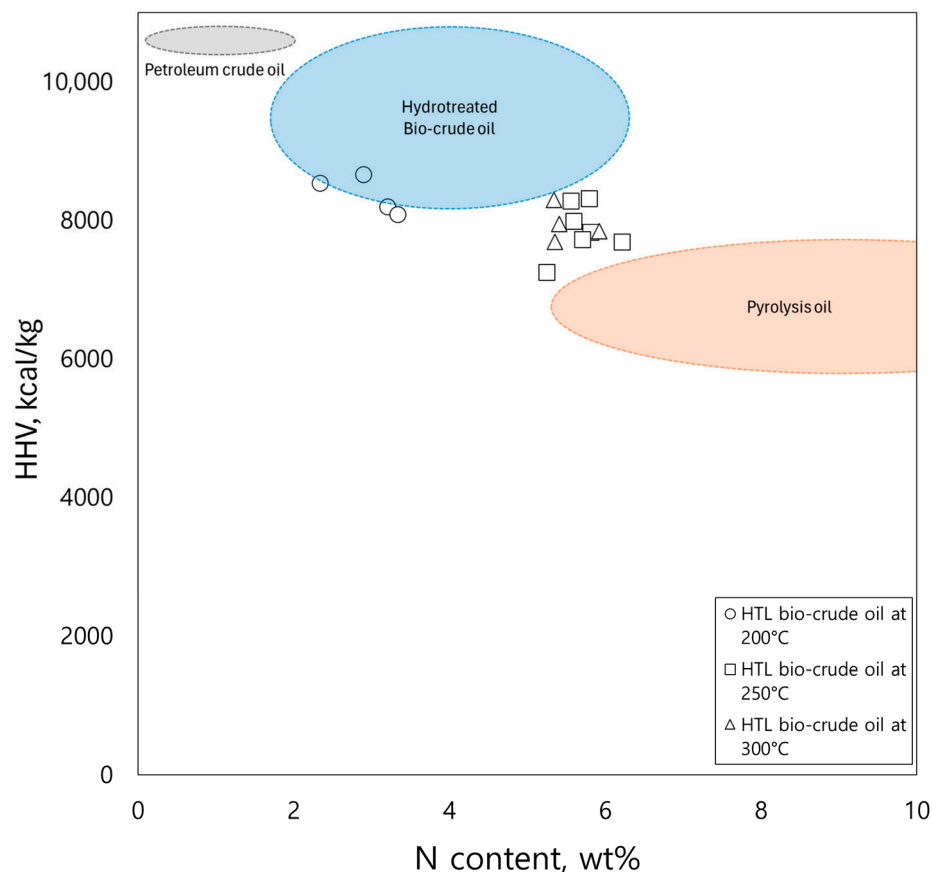


Figure 7. Distribution of nitrogen content and higher heating value (HHV) of HTL bio-crude oils obtained under different operating conditions, compared with literature-reported petroleum crude oil, non-catalytic pyrolysis oil, and hydrotreated bio-crude oil regions.

Bio-crude oils obtained at 200 °C exhibited relatively low nitrogen contents (approximately 2.34–3.34 wt%) and comparatively high HHVs (approximately 8080–8661 kcal/kg). In contrast, bio-crude oils produced at 250–300 °C showed increased nitrogen contents (approximately 5.25–6.22 wt%), whereas their HHVs were not substantially higher than those obtained at 200 °C.

When compared with literature-reported non-catalytic microalgae pyrolysis oil, which shows nitrogen contents of approximately 5.3–12.8 wt% and HHVs of approximately 5830–7740 kcal/kg, the HTL bio-crude oils obtained in this study generally had lower nitrogen contents and higher HHVs [43]. This comparison suggests that the HTL products had more favorable HHV–nitrogen characteristics than untreated microalgae pyrolysis oils. However, the 250–300 °C samples partially overlapped with the lower-nitrogen side of

the pyrolysis oil region, indicating that nitrogen enrichment in the oil phase remains an important limitation under high-temperature HTL conditions.

The relatively high HHVs observed at 200 °C may be associated with the preferential preservation or recovery of lipid- and fatty-acid-derived compounds under mild HTL conditions [45]. Previous studies have reported that low-temperature HTL bio-crude oil from microalgae can exhibit compositions dominated by free fatty acids and that lipids are predominantly converted into free fatty acids below approximately 250 °C [46]. Therefore, although the 200 °C samples appeared more favorable than the 250–300 °C samples from the HHV–nitrogen perspective, this does not necessarily indicate superior fuel-intermediate quality, particularly because energy recovery (ER) also depends on bio-crude oil yield. The ER values, calculated as described in Section 2.2, were 17.02–31.82%, 35.48–51.26%, and 46.52–60.70% for the 200, 250, and 300 °C conditions, respectively. This indicates that the apparent advantage of the 200 °C samples in HHV–nitrogen characteristics was partly offset by their lower bio-crude oil yields. Therefore, the quality of HTL bio-crude oil should be evaluated in conjunction with compound-level compositional characteristics.

Further comparison with petroleum crude oil and hydrotreated bio-crude oil regions provides additional context for evaluating the HHV–nitrogen characteristics of the obtained HTL bio-crude oils. Petroleum crude oil exhibited a much higher HHV region (approximately 10,400–10,800 kcal/kg) and lower nitrogen contents (approximately 0.1–2.0 wt%), clearly distinguishing it from the untreated HTL bio-crude oils [47]. The hydrotreated bio-crude oil region shifted toward higher HHVs (approximately 8170–10,820 kcal/kg), indicating that hydrotreatment can substantially improve the energy density of bio-crude oil [44]. However, its nitrogen-content (approximately 1.69–6.3 wt%), still partially overlapped with that of the untreated HTL bio-crude oils [44]. This suggests that an improvement in HHV after hydrotreatment does not necessarily correspond to a proportional reduction in nitrogen content. Therefore, the fuel-related quality of HTL bio-crude oils should be evaluated not only by bulk indicators such as HHV and nitrogen content, but also by compound-level compositional characteristics, as discussed in the following GC–MS analysis.

3.6. GC–MS Analysis of Compositional Changes in Bio-Crude Oil

Figure 8 presents the relative distribution of major compound groups identified in the bio-crude oil samples under different HTL temperature conditions.

To provide a mechanistic basis for interpreting GC–MS results, the conversion pathways of major microalgal biomolecules during HTL should be considered [35]. Lipids are first hydrolyzed into free fatty acids, which can then undergo decarboxylation, decarbonylation, cracking, and other deoxygenation reactions to form hydrocarbons [48,49]. Proteins decompose into peptides, amino acids, ammonia, and amine-containing intermediates. Meanwhile, carbohydrate-derived intermediates such as carbonyl compounds and furans, can react with amino compounds through condensation and Maillard-type reactions, contributing to the formation of amides and heterocyclic nitrogen compounds [34–36]. Therefore, the compound groups detected by GC–MS should be interpreted as products of parallel and competing reactions among lipid-, protein-, and carbohydrate-derived intermediates, rather than as independent compound classes. In this context, the GC–MS results are discussed as semi-quantitative compositional trends based on normalized relative peak areas, rather than as absolute compound concentrations.

Figure 8 illustrates the temperature-dependent changes in the relative distribution of major compound groups in the bio-crude oil samples. Hydrocarbons and fatty acids generally exhibited opposing trends with increasing temperature. However, since each data point in Figure 8 was obtained under different combinations of pressure and reaction time within the same temperature group, the relative distribution of each compound group

reflects not only the effect of temperature but also the combined influences of pressure and reaction time.

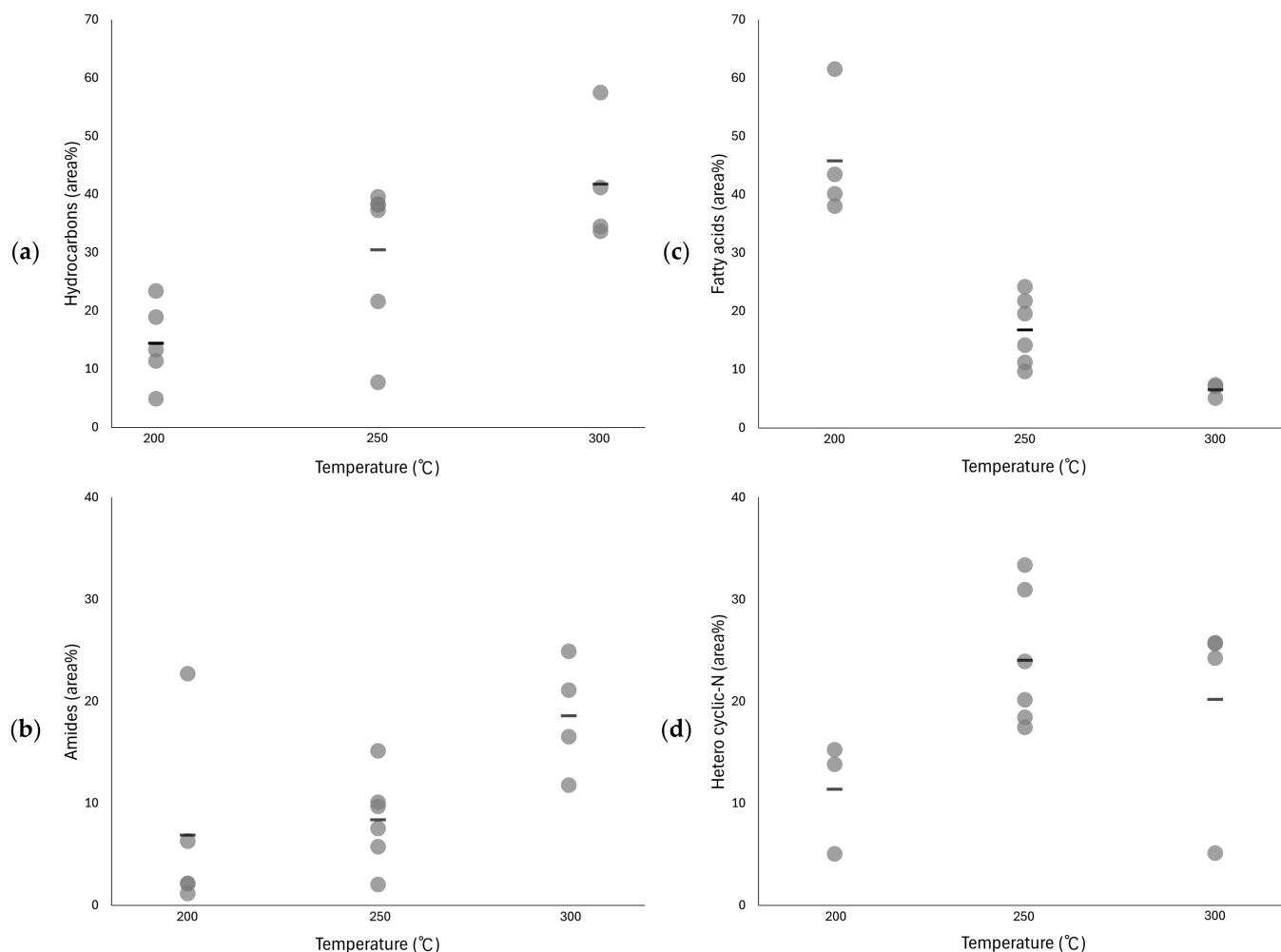


Figure 8. Temperature-dependent changes in major compound groups of bio-crude oil samples revealed by GC–MS: (a) hydrocarbons, (b) fatty acids, (c) amides, and (d) heterocyclic nitrogen compounds. Black horizontal bars indicate the mean relative peak area of the individual runs within each temperature group.

As the HTL temperature increased from 200 °C to 300 °C, the relative abundance of hydrocarbons generally increased (Figure 8a), while the proportion of fatty acids decreased markedly (Figure 8b). These results suggest that lipid-derived compounds were predominantly retained as fatty acids under mild HTL conditions, whereas higher temperatures promoted further deoxygenation of fatty acids, leading to a more hydrocarbon-dominant composition. Previous studies have reported that microalgal lipids can initially decompose into fatty acids during HTL, which may subsequently undergo decarboxylation and decarbonylation reactions to form hydrocarbons [48,49]. Therefore, from a fuel-feedstock perspective, high-temperature conditions appear more favorable for forming hydrocarbon-centered compositions than oxygen-containing, fatty-acid-rich compositions.

For amide compounds, relatively higher average abundances were observed at 300 °C compared to those at 200 °C and 250 °C (Figure 8c). This behavior suggests that increasing HTL severity does not simply eliminate nitrogen-containing compounds in one direction but may also promote their rearrangement or retention as amide-type compounds within the oil phase. Previous studies have identified various fatty amides in algal HTL green crude [34], interpreted as condensation products formed from fatty acids and protein-

derived amines or ammonia. Additionally, studies on hydrothermal reactions of microalgal biomolecules have suggested that amide compounds can be generated through reactions between fatty acids, amino acids, and ammonia under hydrothermal conditions [35]. Thus, amide compounds may be regarded as a nitrogen-containing compound group highly sensitive to changes in HTL reaction severity.

N-heterocyclic compounds increased substantially at 250 °C compared to at 200 °C and remained detectable at 300 °C (Figure 8d). However, while the 250 °C condition exhibited the highest average abundance and largest distribution range, the 300 °C condition showed relatively lower and narrower distributions. The formation of N-heterocyclic compounds can also be associated with secondary reactions between protein-derived amino compounds and carbohydrate-derived carbonyl intermediates, followed by condensation, cyclization, and aromatization [35,36]. This behavior suggests that cyclization and aromatization of nitrogen-containing intermediates were actively promoted near 250 °C, leading to the accumulation of N-heterocyclic compounds. In contrast under the more severe HTL condition at 300 °C, some nitrogen-containing species may have undergone further transformation or redistribution into phases outside the oil phase. Consequently, the GC–MS-detectable fraction of N-heterocyclic compounds did not increase monotonically with temperature.

Previous studies have reported that protein-derived nitrogen during microalgal HTL can be transformed into heterocyclic and aromatic nitrogen compounds such as pyridine and quinoline [36], while nitrogen can also be distributed differently among the bio-crude oil, aqueous, gaseous, and solid phases depending on HTL operating conditions [18]. Recent studies have further suggested that a substantial fraction of nitrogen can be transferred into the aqueous phase during microalgal HTL [33]. Therefore, the differences observed between the 250 °C and 300 °C conditions in the present study may be interpreted in relation to temperature-dependent nitrogen transformation and phase-partitioning behavior during HTL processing.

Within the investigated process range, the 300 °C condition did not completely eliminate nitrogen-containing compounds; however, it appeared to represent the most favorable operating region for simultaneously achieving enhanced bio-crude oil yield and a more hydrocarbon-dominant composition.

Overall, the GC–MS results indicate that the compositional evolution of the bio-crude oils was governed by competing HTL reaction pathways rather than by a simple one-directional conversion. Increasing temperature promoted the conversion of lipid-derived fatty acids into hydrocarbons through deoxygenation reactions [48,49]. In contrast, protein-derived nitrogen did not follow a simple removal pathway but appeared to undergo two competing routes: redistribution of relatively polar nitrogen-containing species into aqueous and other non-oil phases, and retention in the bio-crude oil phase through the formation of amide and N-heterocyclic compounds via condensation, cyclization, and aromatization reactions [18,33–36]. Thus, the persistence of nitrogen-containing compounds at high temperatures reflects the combined effects of nitrogen transformation, secondary condensation reactions, bio-crude oil-phase retention, and phase redistribution during microalgal HTL [18,33–36].

4. Discussion and Conclusions

In this study, the HTL process of *C. vulgaris* was evaluated using a Box–Behnken design (BBD). We analyzed the effects of temperature, pressure, and reaction time on bio-crude oil yield, nitrogen content, and fuel-related properties. Temperature emerged as the primary process variable, strongly influencing both bio-crude oil yield and nitrogen content, while the effects of pressure and reaction time were relatively limited.

Bio-crude oil yield increased significantly with rising temperature, whereas nitrogen content showed a nonlinear response. This highlights that, when determining HTL operating conditions, both yield improvement and changes in the nitrogen content of the produced bio-crude oil must be considered.

Based on multi-response optimization within the experimental range, the compromise optimum HTL condition was identified as 300 °C, 200 bar, and 15 min within. This condition predicted a bio-crude oil yield of 39.85 wt% and a nitrogen content of 5.01 wt%. It should be noted that this condition is not for nitrogen removal, but rather a practical compromise balancing high bio-crude oil yield with nitrogen retention in the oil phase.

Analysis of fuel-related properties confirmed that HTL promoted deoxygenation, as evidenced by lower O/C ratios in the bio-crude oils compared to the raw microalgal feedstock. Compared to literature-reported reference regions, the HTL bio-crude oils exhibited more favorable elemental and HHV–nitrogen characteristics than non-catalytic microalgae pyrolysis oil, though they remained distinctly different from petroleum crude oil. Although samples produced at 200 °C showed relatively favorable HHV–nitrogen characteristics, this does not necessarily indicate superior fuel-intermediate quality, as mild HTL conditions may preserve lipid- and fatty-acid-derived compounds. In contrast, the hydrotreated bio-crude oil region was closer to petroleum crude oil, suggesting that downstream upgrading can improve fuel-related properties, even if nitrogen reduction remains limited. Therefore, the fuel-intermediate quality of HTL bio-crude oil should be evaluated not only using bulk indicators such as O/C ratio, HHV, and nitrogen content, but also in conjunction with compound-level compositional characteristics.

GC–MS analysis revealed that the relative proportion of fatty acids decreased with increasing temperature, while that of hydrocarbons increased. This trend suggests that lipid-derived fatty acids were progressively converted into hydrocarbons through deoxygenation reactions under higher-temperature HTL conditions. In contrast, amides and heterocyclic nitrogen compounds persisted even under high-temperature conditions, indicating that nitrogen-containing compounds are crucial quality-control factors for HTL bio-crude oil. These results also suggest that protein-derived nitrogen does not follow a simple removal pathway but can be retained in the oil phase through secondary reactions forming amides and N-heterocyclic compounds.

While this study provides a systematic evaluation of HTL operating conditions using BBD, several limitations should be considered. Pressure was treated as an operationally controlled condition, and optimization was primarily based on oil-phase responses without complete phase-wise mass balance. Additionally only the center-point condition was replicated, GC–MS results were interpreted semi-quantitatively, and downstream upgrading was not performed. Future studies should therefore include full product distribution analysis and additional replication to further validate the optimized condition. Furthermore, upgrading/refining strategies such as catalytic hydrotreating, denitrogenation, and catalyst screening should be investigated to improve the bio-crude oil for final fuel applications.

Overall, this study demonstrates that the optimization of microalgae-derived HTL bio-crude oil should not be based on yield alone, but must also consider nitrogen retention, fuel-related properties, and compositional evolution. By integrating BBD-based process evaluation with elemental, energy-based, and GC–MS analyses, this study provides a practical basis for selecting HTL operating conditions to produce *C. vulgaris*-derived bio-crude oil as an upgrading-oriented fuel intermediate.

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G.L. and W.C.; writing—review and editing, W.C., S.C. (SoonWoong Chang), Y.A., G.L., B.-H.J., M.S.P., S.H. (Sooyoul Hong), S.C. (Sangkyu Choi) and S.H. (Soyoung Han); visualization, G.L.; supervision, W.C. and S.C. (SoonWoong Chang); project administration, S.C. (SoonWoong Chang) and M.S.P.; funding acquisition, S.C. (SoonWoong Chang). All authors have read and agreed to the published version of the manuscript.

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Abbreviations

The following abbreviations are used in this manuscript:

HTL	Hydrothermal liquefaction
BBD	Box–Behnken design
RSM	Response surface methodology
ANOVA	Analysis of variance
HHV	Higher heating value
GC-MS	Gas chromatography mass spectrometry

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