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A study to investigate the energy recovery potential from different macromolecules of a low-lipid marine *Tetraselmis* sp. biomass through HTL process

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ABSTRACT

This study investigated the hydrothermal liquefaction (HTL) of extracted major macromolecules from *Tetraselmis* sp. biomass. The carbohydrate fraction was first recovered from *Tetraselmis* biomass using pressurized heated water. The crude lipid fraction was then extracted from carbohydrate-free biomass by hexane. The remaining biomass was considered as protein extract. HTL runs were conducted from 275 to 350 °C and 30 min for each extract; the maximum biocrude yields for carbohydrate, lipid, and proteins were obtained at 325, 325, and 350 °C, respectively. Next, HTL runs of these macromolecules were conducted at 350 °C for 10–60 min. The highest biocrude yields from carbohydrate, lipid, and protein extracts were obtained at 45, 20, and 45 min, respectively. The optimal energy recovery, as biocrude, from carbohydrate, lipid, and protein extracts were 41, 85, and 81%, respectively. Therefore, microalgae biomass with low carbohydrate content or carbohydrate-extracted biomass could be used as feedstock for biocrude production.

1. Introduction

Marine microalgal biomass could be an alternative and renewable feedstock for biofuel production [1–3]. Hydrothermal Liquefaction (HTL) has emerged as a promising technology to convert various biomass feedstock in biocrude oils [4–8]. Microalgal biomass is mainly comprised of three major macromolecules, such as protein, lipid, and carbohydrates [9,10]. During the HTL process, these compounds could have different rates of conversion to biocrude [12,13]. Therefore, it would be essential to understand how the major ingredients of any microalgal biomass would convert into biocrude so that an efficient HTL operating system could be developed. There are several kinetic studies of biocrude production from the whole microalgae biomass [14–17]. However, there are a limited number of kinetic studies on converting different types of macromolecules to biocrude. Furthermore, the macromolecule compounds were sourced from different feedstocks (soy protein, glucose, vegetable oils, etc.) [18,19]. The microalgal macromolecules composition varies among strains and the growth conditions, leading to varying biocrude yield and quality [20–22]. Hence, the characteristics of the biocrude from different microalgal macromolecules could assist in selecting growth conditions leading to the production of biomass feedstock with the desired quality for biocrude production. HTL study of individual microalgal macromolecules could also shed light on the loss of some components in the aqueous phase. Therefore, the biocrude conversion rate and yield from the major macromolecules of microalgal biomass need to be investigated.

The annual growth potential of a halotolerant *Tetraselmis* sp. was recently explored in an outdoor raceway tank (size: 200 L) with growth media recycling (Das et al., 2019b). Low energy requirement in harvesting and high biocrude yield made *Tetraselmis* sp. a potential microalgal strain for producing biofuel feedstock (Das et al., 2019a). Therefore, *Tetraselmis* sp. was selected as a model strain for this study. Earlier studies have demonstrated that *Tetraselmis* sp. could efficiently uptake the nutrients (N, P, and other trace metals) from the aqueous phase (Das et al., 2020). Nonetheless, an insight

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into HTL reaction conditions leading to the optimal release of nutrients in the aqueous phase would be very useful for microalgal nutrient recycling.

Earlier studies utilized cornstarch and cellulose as polysaccharides, albumin and soy protein as proteins, castor and sunflower oil as lipids, and whole Nannochloropsis sp. biomass as different feedstocks for HTL studies [23]. In another study, proteins, lipid, and carbohydrate values from *Nannochloropsis* sp., *Scenedesmus* sp., and *Chlorella* sp. were measured, and later the measured values were used to develop yield prediction models for HTL products such as biocrude, aqueous phase, and gas [17,24]. Similarly, measured biochemical metabolite values of *Aurantiochytrium* sp. and *Tetraselmis* sp. were used to solve ordinary differential equations and develop models for studying the HTL product distribution [14,25]. However, each microalgal metabolite fraction (i.e., lipid, protein, and carbohydrate) would have multiple compounds (see supplementary). Therefore, HTL experiment with microalgal extracts could have better representation compared to the HTL of single model compounds – sourced from other feedstocks. Another study reported pretreatment of microalgae at subcritical temperatures for improving the biocrude quality [26]. HTL of individual macromolecules could help understand how each individual macromolecule affects the HTL performance of whole microalgae so that the results obtained from HTL of individual macromolecules could guide in process optimization and improving biocrude yield and quality in the future.

Some of earlier published literature conducted HTL experiments on separately sourced single or binary mixtures of model compounds (i.e., soy proteins, castor oil, glucose, cornstarch; crude protein, and crude polysaccharides from unknown microalgae) to study the effect of reaction parameters on product yield and quality to develop predictive kinetic models for HTL process for a variety of feedstocks [19,27,28]. Whereas, In this research, instead of conducting HTL from differently sourced model compounds and microalgae biomass; a novel multistep pretreatment technique was used to extract individual macromolecules from a marine microalga *Tetraselmis* biomass.

The objective of this study was to understand how the HTL operating conditions could affect biocrude yield and quality, nutrient distribution, and energy recovery from individual microalgal metabolite feedstock and compare with whole biomass. Hence, at first, the macromolecules (i.e., proteins, lipids, and carbohydrates) were extracted from *Tetraselmis* biomass, followed by HTL experiments with the extracted macromolecules at varying reaction temperatures and time durations.

2. Methodology

2.1. Production of Tetraselmis sp. biomass

At first, inoculum of *Tetraselmis* sp. was prepared indoor in 10 L plastic PBRs using artificial light. Later, the cultures from 5, 10 L PBRs were inoculated into a 1000 L raceway tank. Cultures from 2, 1000 L raceway tanks were used to inoculate one 25,000 L raceway pond. After 7 days of growth in the 25,000 L raceway pond, 10,000 L was transferred to 100,000 L (i.e., 500 sq. m) raceway pond. Modified Guillard f/2 medium was used to grow *Tetraselmis* sp. in all cases [29]. After 8 days of cultivation, more than 3000 L culture was harvested using a pilot-scale cross-flow unit. The slurry obtained after the cross-flow filtration was further concentrated using a US Ultrafiltermaxx G + 6000 centrifuge. The details of *Tetraselmis* sp. cultivation and the biomass harvesting were reported elsewhere [29]. The centrifuged biomass was then dried using a pilot-scale rotary drum dryer by blowing hot air through the drum (see supplementary). The temperature of the hot air was maintained at 60 ± 2 °C.

2.2. Characterization of the macromolecules

The ash content in the dried *Tetraselmis* sp. biomass was determined by knowing the loss of weight of a pre-weighed biomass sample at 500 °C for 6 h in a SNOL-80/1150 muffle furnace. Cellular protein, carbohydrate, and lipid fractions were determined using modified versions of the Lowry method, phenol-sulfuric acid method, and Bligh and Dyer method, respectively; the details of the macromolecules characterization were reported earlier [30–33]. All the macromolecules were reported on an ash-free dry weight basis.

2.3. Extraction of macromolecules

The dried biomass was made powder using a bench-top grinding machine.

2.3.1. Two-step carbohydrate extraction process

The powdered biomass was mixed with DI water, the mixture was heated for 1 h inside a pressure cooker using a hotplate – maintained at 140 °C. Next, the slurry was centrifuged to separate the biomass, and the carbohydrate-containing dark brown aqueous layer was collected (**see supplementary**). The pelletized centrifuged biomass was separated from the aqueous layer and used as a feedstock for carbohydrate extraction using the same procedure for one more time. After two consecutive processing, the carbohydrate concentration in the mixed solution was 17.5 g/L; this solution was then further concentrated by evaporation to obtain a carbohydrate concentration of 148 g/L. Next, DI water was added to reach a carbohydrate concentration of 1 g/7 mL (i.e., 142.8 g/L). The 1g/7 mL carbohydrate concentration was desirable to conduct HTL with carbohydrate extract having a concentration of 14.2%, as earlier reported literature showed that HTL feedstock concentration should be in the range of 10–16% [34,35].

2.3.2. Lipid extraction

The separated biomass was then dried again in the oven at 60 °C for 24 h and made powder; the lipid fraction from the powdered biomass was then extracted by hexane using a Soxhlet apparatus. 500 mL solvent was used per 100 g of powdered biomass; the Soxhlet was operated for 12 h per batch. All the hexane layers were pooled together, and the lipid fraction was separated from hexane using a Buchi rotary evaporator (R - 100). After separating the hexane layer, the biomass was again dried in the oven to remove the residual hexane. The biomass obtained from this layer was considered as protein fraction.

2.4. HTL experiment

All the HTL experiments were conducted in triplicates in 10 mL size Swagelok unions (reactors) with 7 mL working volume. After loading 1 g feedstock (microalgal macromolecules and the whole biomass) and 6 mL deionized water in the reactors, the reactors were kept inside an oven at predetermined temperatures (275–350 °C) for 30 min. Next, for each metabolite fraction and the whole biomass, HTL experiments were conducted at 10–60 min at 350 °C. After completion of the HTL reaction, the Swagelok reactors were removed and quenched under running tap water. Later Dichloromethane (CH_2Cl_2) was added to the HTL reaction mixture to form two phases; the upper phase contained aqueous phase liquid, and the lower layer contained the biocrude – dissolved in dichloromethane. Dichloromethane from the lower organic phase was recovered and evaporated at 40 °C; the remaining liquid product was defined as biocrude oil. The biocrude oil yield was calculated on dry weight basis using equation (1).

$$Bicorude\ oil\ yield\ (wt\ \%) = \frac{W_{biocrude\ oil}}{W_{feed}} \times 100 \quad (1)$$

2.5. Characterization of the aqueous phase byproducts

The concentrations of total nitrogen (TN) and total organic carbon (TOC) in the aqueous phase liquid (APL) were determined using LCK 138 and LCK 385 HACH kits and HACH DR3900 spectrophotometer detailed procedure for TN and TOC are given in the supplementary file of the manuscript.

2.6. Characterization of biocrude oil

Biocrude oil was characterized using an Agilent technologies GC system 7890A coupled with a 5973 network mass selective detector. The injector temperature was maintained constant at 300 °C. Autosampler was used for injecting 1 μL sample (in splitless mode) onto the GC column (30 m × 250 μm x 0.25 μm Rxi 5sil MS column). Helium was used as carrier gas with a flow velocity of 1.67 mL/min and 15 psi pressure. The oven temperature was held at 60 °C for 2 min, then was increased at a rate of 6 °C/min until it reached 300 °C, where it was held for 20 min. The different compounds within the gas stream were ionized using an ion source. The mass spectrometer was operated in MS-Scan mode, various compounds in the biocrude samples were identified using the NIST98 mass spectral database and were later grouped as alkanes, alkenes, and heterofunctional compounds.

2.7. Determination of the higher heating value (HHV)

A flash 2000 CHNS analyzer was used to determine C, H, and N in test samples. Oxygen was calculated by subtracting the total C, H, and N from 100 [26,36]. Sulfur levels were below instrument detection limits; therefore, S content was not included in calculating HHV. The calorific values of the feedstocks and biocrude samples were determined by their respective higher heating values (HHV). The HHV value was calculated using the following Dulong's formula [37].

$$Higher\ heating\ value\ \left(\frac{MJ}{kg}\right) = 0.338\,C + 1.428\left(H - \frac{O}{8}\right) + 0.095 \quad (2)$$

2.8. Determination of energy recovery

The energy recovery, as biocrude oil, from the feedstocks for HTL runs at different times were calculated. The energy recovery (ER, %) was determined using the following equation:

$$ER\ (\%) = Biocrude\ yield\ (\%) \times \frac{HHV\ (biocrude)}{HHV\ (feedstock)} \quad (3)$$

3. Results and discussion

3.1. Metabolite extraction process

The salinity of the *Tetraselmis* sp. culture was 4.2% NaCl during the harvesting procedure; the culture was free from other microalgae or cyanobacteria during the harvesting time. Cross-flow-filtration was used to process 3544 L culture and obtain a concentrated slurry of 108 L [29]. The centrifuged biomass had a solid content of 24.6%; the dry biomass obtained from the rotary drum dryer had a solid content of 93%. Ash content in the dried biomass was 23%. On a ash free dry weight basis, the harvested *Tetraselmis* sp. biomass had 32.1 ± 2.3%, 18.8 ± 0.7%, and 40.7 ± 1.8% protein, lipid, and carbohydrate, respectively. From 1000 g dry biomass, the obtained carbohydrate, lipid, and protein extracts were 280 g, 110 g, and 241 g; the remaining fraction would account for ash and loss (Fig. 1). The carbon, nitrogen, hydrogen, and oxygen contents in the *Tetraselmis* sp. biomass and its three extracts were compared in Table 1.

The combined carbohydrate recovery for the two consecutive pressurized water extractions was 68%. The extraction efficiency of carbohydrates from microalgal biomass using a hot water extraction process was reported to be in the range of 30–40% [38]. The higher efficiency observed here could be explained by two consecutive extractions and the inclusion of pressure. The molar ratio of carbon to oxygen in the carbohydrate extract was 1:1.16; however, the typical value of carbon to oxygen ratio in the carbohydrate would be 1:1. Increased oxygen content in carbohydrates could be due to hydrolysis, dehydration of polymeric carbohydrates into monomers. Moreover, during the extraction process, these monomers could undergo cyclization and aromatization to form aromatics having oxygen-containing functional groups [19,39]. The pressure cooker was used to maintain subcritical water (or superheated water) for extracting carbohydrates; the subcritical water could also have initiated hydrolysis of some protein fractions into water-soluble chemicals [40]. The carbohydrate extract had a nitrogen concentration of 8 g/L, which accounted for 31% of cellular nitrogen content. Chen et al. (2017) explored a two-stage sequential hydrothermal liquefaction (SEQHTL) where the first phase was conducted at a lower temperature (i.e., 163 °C) followed by a second phase at elevated temperature. The concentrations of sugar and total nitrogen in the aqueous phase liquid, obtained from the 1st phase of SEQHTL, were 7.5 and 3.7 g/L, respectively [41].

The lipid extract could have some impurities in the form of other metabolites. Microalgal lipids are mostly triglycerides – comprised of carbon, hydrogen, and oxygen, although the phosphorus could be found in some membrane-bound phospholipids. In the lipid extract, 1.2% of nitrogen was present, which could be contributed by hexane soluble pigments and other metabolites. Kanda et al. [42] also reported that crude lipid extract from microalgae had 2.62% nitrogen in addition to other trace metals [43]. Cheng et al. (2011) reported the extracted crude lipid from microalgae could have a high content of impurities, and the fraction of impurities could vary based on the solvent used [44].

In general, nitrogen content in the protein compounds is around 15%. However, the nitrogen content in the protein extract was much below (i.e., 9.2%). Because of the incomplete extraction of carbohydrate and lipid, the remaining compounds, mostly nitrogen-free, could have reduced the nitrogen content in the protein extracts. Similarly, a fraction of the nitrogenous compounds were separated in the carbohydrate fraction, reducing the nitrogen content in protein extract. 45.2% of the cellular ash content was associated with the combined carbohydrate extracts. Due to the separation of carbohydrate and lipid fraction from the biomass, the ash content in the protein fraction was slightly higher (i.e., 25.7%) than the original biomass feedstock.

The HHV of the whole biomass was 20.15 MJ/kg, whereas the corresponding HHV of protein, carbohydrate, and lipid extracts were 21.8, 13.8, and 37.7 MJ/kg, respectively. Kanda et al. (2012) reported that HHV of microalgal lipid extract was 33.8 MJ/kg [43]. A slightly higher HHV value of lipid extract was obtained in this study since some of the impurities (and perhaps having low HHV) were

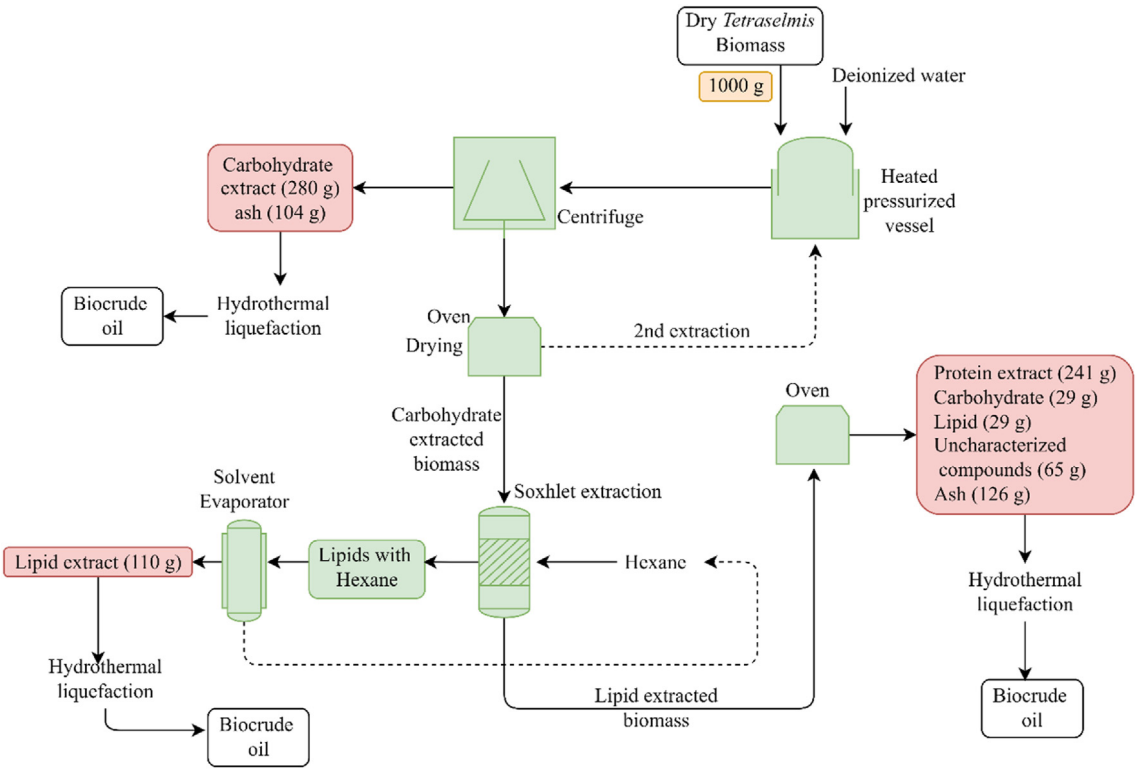

Fig. 1. A schematic of extracting different major macromolecules of *Tetraselmis* sp. biomass.

Table 1
Elemental analysis of biomass and extracted macromolecules.

Feedstock	C	H	N	O[a]	HHV (MJ/kg)
Biomass	48.9 ± 1.9	7.2 ± 0.7	6.5 ± 1.1	37.3 ± 2.9	20.2 ± 2.49
Protein extract	49.2 ± 1.2	7.9 ± 1.2	9.2 ± 1.0	33.7 ± 2.4	21.8 ± 2.74
Carbohydrate extract	42.2 ± 0.6	5.5 ± 0.7	5.6 ± 0.9	46.8 ± 2.3	13.8 ± 1.64
Lipid extract	74.2 ± 1.4	10.6 ± 1.6	1.1 ± 0.3	14.1 ± 2.6	37.7 ± 3.28

[a] by difference and ignoring S content (profiles of protein, lipid, and carbohydrate of *Tetraselmis* sp. could be found in supplementary).

partitioned in carbohydrate extract, which could have otherwise associated with the lipid extract. It has been reported that the HHV of crude protein and crude carbohydrate extracts of low lipid microalgae were 21.6 and 11.5 MJ/kg, respectively [19].

3.2. The effect of HTL reaction temperature on the biocrude yield from the extracted macromolecules

The biocrude yields from *Tetraselmis* sp. biomass and its extracted macromolecules, for the temperature range 275–350 °C, are shown in Fig. 2. For the lipid and carbohydrate extracts, there was an increasing trend of biocrude yield with an increase in HTL operating temperature till 325 °C. After 325 °C, the biocrude yield for whole biomass and protein content increased while the biocrude content decreased for lipid and carbohydrate; it was reported that at mild hydrothermal treatment (HTT) reaction conditions (i.e., 250 °C or less), the lipid and algaenans mostly contributed to the HTT oil, whereas elevated reaction temperatures (300 °C and above) promoted the conversion from carbohydrate and protein [45]. However, the major compounds of lipid derived HTT oil could be classified mainly as lipid, which could also be extracted without HTT; furthermore, the biocrude oil had unconverted lipids. Hence, higher temperature would be essential for producing biocrude oil from different feedstocks, especially from low lipid biomass

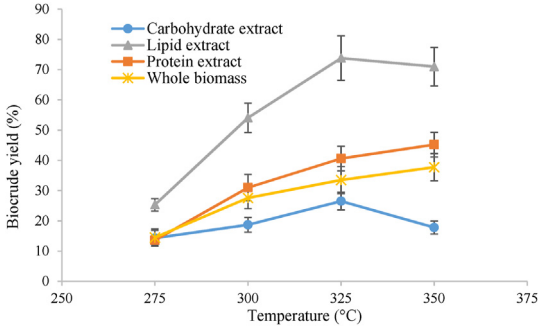

Fig. 2. Biocrude yield for the extracted macromolecules and the whole biomass at 275–350 °C for 30-min reaction time.

feedstock. The maximum conversion of whole biomass (37.73%) and protein extract (45.2%) was at 350 °C, whereas the maximum biocrude yield for lipid (73.8%) and carbohydrate extract (26.5%) was at 325 °C. In this study, the biocrude yield from the ash-free biomass of *Tetraselmis* sp. was 48.9%, which was consistent with earlier reports from the author's group.

Yang et al. (2015) reported that the biocrude yield from crude polysaccharides at 300 °C was low (i.e., 5%); whereas, in this study,

a biocrude yield was 18.7% at 300 °C; in this study higher biocrude yields could be due to the presence of cellular proteins in the carbohydrate extract. At 350 °C, the biocrude yield from a model carbohydrate compound (i.e., lactose) was 7% [18]. Brilman et al. (2017) studied the HTL of sugar extracted beet pulp as a carbohydrate-rich feedstock; the biocrude yield increase as the temperature increased from 250 °C to 350 °C. A low biocrude oil yield of 8.5% was reported for pure glucose feedstock at 340 °C; however, the biocrude yield increased when the feedstock was a mixture of carbohydrates and protein (Qiu et al., 2019). In this study, water-soluble carbohydrate extract (included nitrogenous compounds) was used, and the maximum biocrude yield from carbohydrate extract was 22% (i.e., 16.3%, considering the ash in the extract). Since there were as much as 40% other non-carbohydrate compounds (mainly proteins) in the carbohydrate extract, the biocrude yield in this study was higher than the biocrude yield obtained from model carbohydrate compounds – reported earlier. In addition, the increased biocrude yield from carbohydrates could be due to the occurrence of Maillard's reaction leading to the formation of pyrazine derivatives from degraded glucose. Similar biocrude yields were reported for carbohydrate and protein mixture [46,47]. For a protein feedstock (i.e., lysine) and protein-carbohydrate feedstock (lysine + lactose), an increase in HTL operating temperature (range: 250–350 °C), the biocrude oil yield also increased [18]. In this study, the biocrude yields for protein extract and whole biomass increased with increasing HTL operating temperature (275–350 °C); similarly, in another study, for soy protein concentrate (i.e., 75% protein), the biocrude yield increased for an increase in HTL operating temperature (200–350 °C) [48]. In another study, the biocrude yield (i.e., 46%) from *Tetraselmis* sp. was obtained at 350 °C [14]. Valdez et al. (2014) reported that the highest biocrude yield for *Chlorella protothecoides* and *Scenedesmus* sp. were also obtained at 350 °C [17]. Similarly, typical HTL operating temperature studied for other microalgal biomass feedstock was in the range of 300–350 °C, and there was a trend of increasing biocrude yield with the increase in temperature [46,49,50]. Furthermore, since the protein extract fraction was almost half of the biomass and the maximum biocrude yield from protein extract was obtained at 350 °C, for this kinetic study, 350 °C was selected at the fixed operating temperature.

Lu et al. (2018) reported that the biocrude yield from sunflower oil at 350 °C and 30 min of HTL duration was 85% [47]. Similar higher biocrude yields were obtained from sunflower oil (i.e., 80%), palmitic oil (approx. 60%) [11,18]. In this study, 71% biocrude yield was obtained from lipid extract at 350 °C and 30-min HTL reaction time. HTL of low lipid and high protein-containing microalgae produced a maximum biocrude yield of 31%. In contrast, HTL of high lipid and low protein-containing microalgae produced a biocrude yield of 57.2% at 330 °C for 30 min of HTL duration, indicating liquefaction lipids is much higher even at lower reaction temperatures [21]. Furthermore, biocrude yields for high lipid-containing microalgae decreased with increasing reaction holding time and temperature. With increasing temperature, the lipid fraction could crack into carbon dioxide and ammonia; the microalgal membranes could contain glycerophospholipids with N group bonds; therefore, the cracking of glycerophospholipid could have resulted in ammonia formation [51,52]; or re-polymerize into solid coke products, thereby reducing the biocrude yields at reaction temperatures higher than 350 °C.

3.3. The effect of HTL duration on the biocrude yield from the extracted macromolecules

The HTL operating temperature and holding time would influence the biocrude yield and quality. At a lower temperature, a higher holding time might be required; however, for higher operating temperatures, a relatively shorter holding time would be needed [50]. In this study, higher temperatures (i.e., 325–350 °C) and increased reaction time of (i.e., 30–45 min) favored liquefaction-type reactions; further increase in temperature and reaction time could have promoted gasification – reducing liquefaction yields. The biocrude yields from different extracted macromolecules and whole biomass feedstocks at 350 °C for 10–60 min HTL duration are shown in Fig. 3. The HTL runs in this study didn't have a preheating, and hence the biocrude yield at 10 min of HTL duration was the lowest. The biocrude yield from lipid was maximum (i.e., 77%) for 20 min HTL holding time; as the HTL holding time increased, the biocrude yield got reduced. On the contrary, Teri et al. (2014) observed little or no difference in biocrude yield from sunflower oil feedstock at 350 °C over an HTL duration of 10–90 min [23]. The yield from carbohydrate fraction was the lowest for any duration of HTL; at 45 min, the maximum (i.e., 22.4%) biocrude was obtained for carbohydrate. The biocrude yield from the protein extract increased for an increase in HTL duration within 10–45 min; as the HTL duration increased further, there was a small decrease in biocrude yield. Similarly, for soy protein concentrate, there was a general trend of an increase in biocrude oil for an increase in HTL duration (i.e., 10–60 min) for the temperature range 200–350 °C [48]. The biocrude yield from microalgae biomass increased from 11.05% to 37.5% when the HTL duration increased from 10 to 30 min; as the HTL duration increased further, the biocrude yield continued to decrease slightly. In contrast to the results of this study, Vo et al. (2017) reported that at 350 °C, the biocrude yield from *Tetraselmis* sp. was in the range of 42–46% for a time duration of 10–60 min; however, at lower temperatures, an increase in HTL duration increased biocrude yield [14]. The difference in macromolecules compositions between the two *Tetraselmis* biomass feedstocks and the heating procedure (e.g., furnace vs. fluidized bed) could also provide different yields. Earlier studies on whole microalgal biomass reported that extended HTL period (i.e., >30 min) could simultaneously decompose some compounds and repolymerize various compounds to high-molecular compounds - thereby reduced the biocrude yield [49,53]. Valdez et al. (2014) obtained optimum biocrude oil yields at 350 °C from *Chlorella protothecoides* and *Scenedesmus* sp. for 20 and 60 min, respectively [17].

3.4. Composition of biocrude samples for different HTL holding times

Due to the mild temperature of the GC column (<300 °C), the GC-MS technique could characterize 30–50% components in biocrude oil; it has been previously reported that at temperatures under 300 °C only a fraction (e.g., ≤50%) of the biocrude

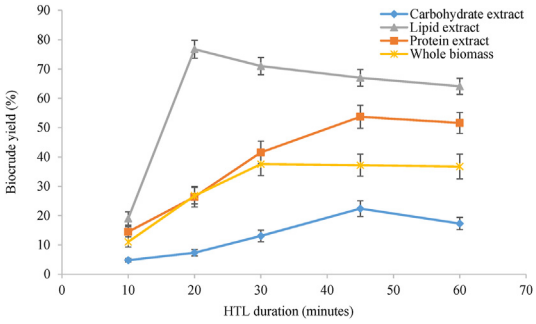

Fig. 3. Biocrude yield from different microalgal macromolecules for HTL reaction time of 10–60 min.

components would volatilize and hence could be detected using a GC-MS [54]. Therefore, residual heavy components of biocrude oil with boiling points greater than 300 °C could not be detected by GC-MS technique. For 10 min of HTL reaction time, the number of compounds in the biocrude samples, obtained from all the feedstocks, were the lowest (i.e., 9–39) (Fig. 4a). For the lipid feedstock, a maximum of 65 compounds was detected at 30 min of reaction time; as the time increased, the lesser number of compounds was detected in the biocrude samples. For carbohydrate feedstock, the number of compounds in the biocrude samples was lower (i.e., 33–58) for the HTL time duration of 10–60 min. For the protein feedstock, the maximum number of compounds in the biocrude sample was found at 45 min of reaction time. For the carbohydrate derived biocrude samples, the alkane compounds were 11–54%, the alkenes were 1.2–10.8%, and the remaining fraction was composed of aromatics and hetero-functional compounds. The biocrude samples, derived from other feedstocks, had much lower alkanes and alkenes compounds compared to aromatics and hetero-functional compounds.

In biocrude samples, different types of compounds, such as hydrocarbons, aromatics, nitrogenated, and oxygenated compounds were identified (Fig. 4b). For whole biomass and protein extract feedstock, a maximum of 173 and 145 compounds were identified at 45 min, whereas a maximum of 65 and 58 compounds were identified within the biocrude samples derived from lipids and carbohydrate macromolecules. For an HTL holding time of 10 min, the maximum area percentage of 48.3 and 48.49% were identified as alkanes for the biocrude samples - obtained from carbohydrates and proteins, respectively; this indicated that lower temperatures could lead to greater alkane formation at optimized reaction temperatures for carbohydrate and protein feedstocks. For carbohydrates, as the HTL duration increased to 60 min, the area percentage for alkanes reduced to 9.2%; simultaneously, there was an increase in aromatic and heterofunctional compounds. At 20 min HTL reaction holding time, a maximum area percentage of heterofunctional and aromatic compounds was identified within biocrude samples derived from whole biomass (85.4%), lipids (97.4%), and protein (81.2%). Conversely, the maximum area percentage of heterofunctional and aromatics compounds in biocrude derived from carbohydrates was 89.7% - obtained at 60 min HTL holding time.

HTL of protein fraction resulted in the formation of N containing compounds such as piperidine, pyridinones, caprolactum, methyl-pyrazines, and amines; these compounds have previously been reported to be derived from the breakdown of various amino acids present within the protein fraction [5]. In the HTL process, various reactions like deamination, lactamization, and cyclization could break down amino acids into various N-containing compounds [18]. In this study, the amino acids from proteins got converted to organic acids (propionic acid, benzoic acid, carbamic acid) and amine group-containing compounds (1-Naphthalenamine, 1-dibenzofuranamine, 4-Cycloocten-1-amine) due to deamination and decarboxylation reactions. With increasing reaction time, the N containing amine derivatives increased in biocrude – obtained from protein fraction of *Tetraselmis* sp. biomass.

Biocrude obtained from HTL of carbohydrate fraction showed the presence of furans, phenols, acids, and aldehydes. Similar compounds were also identified from the HTL of carbohydrates conducted within temperatures ranging from 340 to 400 °C [5]. Interestingly, a large number of alkane fraction ranging from C_4 – C_{24} resulted from HTL of carbohydrate extract of *Tetraselmis* sp. biomass; it has been previously reported that carbohydrates could be converted to C_7–C_{15} alkanes by dehydration, aldol condensation, and hydrogenation reactions [55]. In this study, the formation of alkanes from carbohydrates could have occurred due to dehydration, aldol condensation, and hydrogenation reactions; as the reaction time increased to 60 min, the alkane formation was dramatically reduced due to decarboxylation reactions leading to the formation of coke and carbon dioxide [19].

The biocrude obtained from HTL of lipids resulted in maximum peak areas of 20.9 and 11.9% for alkanes and alkenes, such as fatty acid esters, fatty acid alcohols, and fatty amines were also found in lipid-derived biocrude samples. Some fatty acid and their derivatives were butanoic acid, hexadecenoic acid, lauric acid, heptadecanoic acid, hexadecenoic acid methyl ester, etc.; alcohols such as hexanol, decanol, etc. were also formed. Lipids are composed of fatty acids and glycerol; it has been reported that during the HTL process, the glycerol portion of lipid could be converted to alcohols, aldehydes, ketones, and long-chain hydrocarbons [56]. In this study, long-chain hydrocarbons such as Octacosane ($C_{28}H_{58}$) and

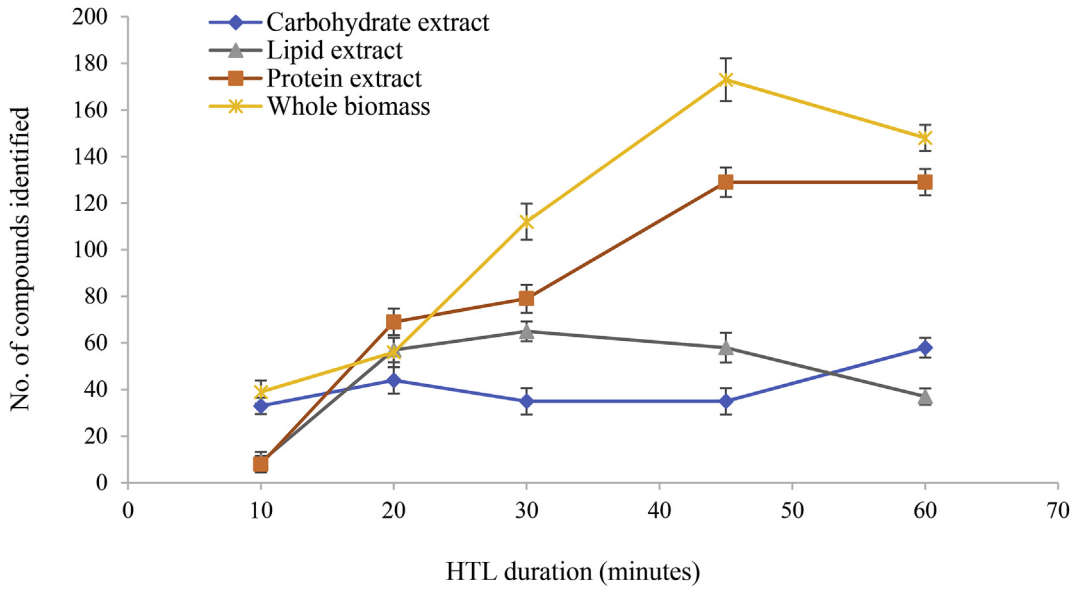

Fig. 4a. No of compounds identified by GC-MS in biocrude obtained from HTL of different microalgal macromolecules for the reaction time of 10–60 min.

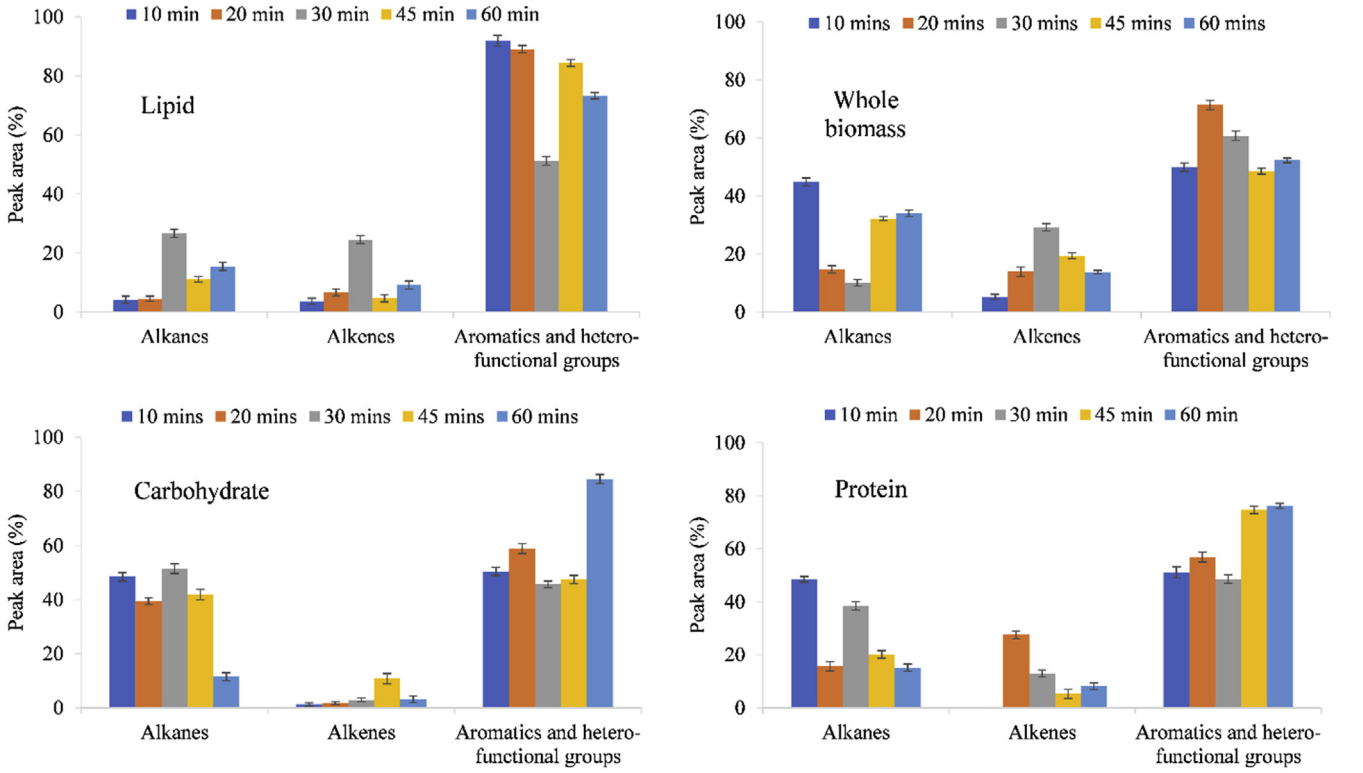

Fig. 4b. Types of compounds identified in the biocrude samples, obtained from the HTL of *Tetraselmis* biomass and its different extracts at 350 °C for the reaction time of 10–60 min.

Tetracontane ($C_{40}H_{82}$) were found in the biorude samples obtained from lipid extract feedstock.

Biocrude derived from whole biomass contained a mixture of N-heterocycles, organic acids, aldehydes, ketones, pyrroles, phytols, porphyrin ring hydrocarbons, fatty acids, and its esters. N- heterocycles could have resulted from Maillard's reaction, whereas other components could have resulted from deamination, dehydration, decarboxylation, and dimerization reactions. The presence of pyrrole, phytols, and porphyrin type hydrocarbons could have been formed from the chlorophyll molecule present in the *Tetraselmis* sp. biomass.

The hydrocarbons in biocrude sample obtained from whole biomass had paraffin lowest being Ethane (C_2H_6) to the highest being Tritetracontane ($C_{43}H_{88}$); whereas the lowest carbon paraffin from lipid extract derived biocrude was Butane (C_4H_{10}); and tetracontane ($C_{40}H_{82}$) being the highest carbon-containing paraffin; ($C_{44}H_{90}$) was the highest carbon-containing paraffin in biocrudes from protein and carbohydrate extracts. As shown in Table 2 biocrude from all the macromolecular extracts contained paraffins that ranged from (C_8–C_{16}); similar range hydrocarbons are needed in bio-jet fuel [57]. Additionally, as shown in Table 2 and the supplementary file, the biocrudes from whole biomass and extracted macromolecules had alkenes and heteroatom-containing compounds; to improve the biocrude quality; further hydrotreatment of biocrudes would be needed for deoxygenation, denitrogenation, and desulfurization; followed by fractional distillation to obtain products of interest (biogasoline, biojet fuel, biodiesel and renewable lubricants).

3.5. The effect of HTL duration on the characteristics of APL

The concentrations of total nitrogen in the aqueous phase for all the feedstocks are shown in Fig. 5a. Nitrogen content in the lipid feedstock was very low, and therefore, the concentration of TN in APL derived from lipid extract was low. The maximum partitioning of nitrogen from the lipid feedstock to the APL was 27%, obtained at 20 min (see supplementary). Nitrogen content in the carbohydrate feedstock was 5.95%. For the HTL of carbohydrate extract, not only the biocrude yields were low, but also a small fraction of the nitrogen (i.e., 6.8–17.3%) ended up in the biocrude; therefore, the bulk of the nitrogen in the feedstock (i.e., 68–88%) remained in the APL. However, with an increase in HTL reaction time, the APL's TN value decreased; this was possibly due to the formation of gaseous products and association in the biocrude. The concentration of TN in the protein extract - derived APL increased with an increase in HTL reaction time; the partitioning of feedstock nitrogen to the APL was in the range of 8–56%. With increasing HTL reaction time, reactions such as lactamization, cyclization, and deamination occur. These reactions breakdown amino acids into various nitrogenous compounds; therefore, at prolonged HTL reaction time, higher amounts of nitrogenous compounds could have been formed. These nitrogenous compounds could then get partitioned either in the biocrude fraction or could in the HTL aqueous phase; thereby, increasing the total nitrogen over increasing time, as shown in Fig. 5a. 7For the whole biomass, TN concentration in the APL increased with the increase in temperature from 10 to 30 min; a further increase in duration until 60 min had little or no effect on the APL's TN concentration. The partitioning of nitrogen in the APL from the whole biomass was 22–46%.

The concentration of TOC in the APL – derived from HTL of different macromolecules was shown in Fig. 5b. TOC concentration in carbohydrate derived APL reduced with an increase in HTL reaction time. TOC in the carbohydrate -derived APL was the sum of unreacted feedstock and the derivative of the feedstock compounds that were converted to biocrude; since the biocrude yield from carbohydrate feedstock increased over time, TOC value in the APL

Table 2
Hydrocarbons in biocrude oil derived from HTL of whole biomass, lipids, proteins and carbohydrates.

Macromolecule	C1–C5	C6–C15	C15+
Whole biomass	Ethane (C_2H_6) Cyclopropane (C_3H_6) Cyclopentane (C_5H_{10})	Hexane (C_6H_{14}) Cyclohexane (C_6H_{12}) Octane (C_8H_{18}) Nonane (C_9H_{20}) Dodecane ($C_{12}H_{26}$) Undecane ($C_{11}H_{24}$) Tetradecane ($C_{14}H_{30}$) Pentadecane ($C_{15}H_{32}$) Benzene (C_6H_6) Hexene (C_6H_{12}) 1-Nonene (C_9H_{18}) 1-Undecene ($C_{11}H_{22}$)	Hexadecane ($C_{16}H_{34}$) Heptadecane ($C_{17}H_{36}$) Octadecane ($C_{18}H_{38}$) Nonadecane ($C_{19}H_{40}$) Eicosane ($C_{20}H_{42}$) Heneicosane ($C_{21}H_{44}$) Docosane ($C_{22}H_{46}$) Tricosane ($C_{23}H_{48}$) Tetracosane ($C_{24}H_{46}$) Nonacosane ($C_{29}H_{60}$) Tritetracontane ($C_{43}H_{88}$) 2-Hexadecene ($C_{16}H_{32}$) 1-Nonadecene ($C_{19}H_{38}$) 1-Octadecene ($C_{18}H_{36}$)
Lipids extract	Butane (C_4H_{10}) Cyclopentane (C_5H_{10})	Cyclooctane (C_8H_{16}) Octane (C_8H_{18}) Nonane (C_9H_{20}) Undecane ($C_{11}H_{24}$) Tetradecane ($C_{14}H_{30}$) Nonadecane ($C_{19}H_{24}$) Benzene (C_6H_6) Cyclohexene (C_6H_{10}) Undecene ($C_{11}H_{22}$) Pentadecene ($C_{15}H_{30}$)	Hexadecene ($C_{16}H_{32}$) Heneicosane ($C_{21}H_{44}$) Octacosane ($C_{28}H_{58}$) Tetracontane ($C_{40}H_{82}$)
Proteins extract	Ethane (C_2H_6) Cyclopentane (C_5H_{10}) Cyclopropane (C_3H_6)	Cyclohexane (C_6H_{12}) Octane (C_8H_{18}) Cyclooctane (C_8H_{16}) Nonane (C_9H_{20}) Decane ($C_{10}H_{22}$) Undecane ($C_{11}H_{24}$) Cycloundecane ($C_{11}H_{30}$) Dodecane ($C_{12}H_{26}$) Tridecane ($C_{13}H_{28}$) Tetradecane ($C_{14}H_{30}$) Pentadecane ($C_{15}H_{30}$) Benzene (C_6H_6) 1-Hexene (C_6H_{12}) 2-Octene (C_8H_{16})	Hexadecane ($C_{16}H_{34}$) Heptadecane ($C_{17}H_{36}$) Octadecane ($C_{18}H_{38}$) Heneicosane ($C_{21}H_{44}$) Tricosane ($C_{23}H_{48}$) Pentacosane ($C_{25}H_{52}$) Tetratetracontane ($C_{44}H_{90}$) 2-Heptadecene ($C_{17}H_{34}$) Eicosene ($C_{20}H_{42}$)
Carbohydrates extract	Butane (C_4H_{10})	Octane (C_8H_{18}) Decane ($C_{10}H_{22}$) Undecane ($C_{11}H_{24}$) Dodecane ($C_{12}H_{26}$) Tridecane ($C_{13}H_{28}$) Tetradecane ($C_{14}H_{30}$) Pentadecane ($C_{15}H_{30}$) Hexadecane ($C_{16}H_{34}$) Heptadecane ($C_{17}H_{36}$) Benzene (C_6H_6) 1-Hexene (C_6H_{12}) 2-Heptene (C_7H_{14}) 1-Decene ($C_{10}H_{20}$) 5-Undecene ($C_{11}H_{22}$)	Eicosane ($C_{20}H_{42}$) Docosane ($C_{22}H_{46}$) Tricosane ($C_{23}H_{48}$) Tetracosane ($C_{24}H_{46}$) Triacontane ($C_{30}H_{62}$) Hentriacontane ($C_{31}H_{64}$) Dotriacontane ($C_{32}H_{66}$) Hexatriacontane ($C_{36}H_{74}$) Tetratetracontane ($C_{44}H_{90}$)

reduced over time. Nevertheless, at least 65% of the carbon in the carbohydrate extract remained in the APL for the HTL duration of 10–60 min. On the contrary, an increased number of water-soluble compounds could have formed during the HTL of lipid feedstock over time, and hence the TOC value in the APL increased over time. For the protein feedstock, TOC concentration in the APL increased till 30 min, and then it reduced over time.

3.6. The effect of HTL duration on energy recovery

The higher heating values (HHV) of the biocrude samples, obtained from the HTL kinetic study of *Tetraselmis* sp. macromolecules, are shown in Fig. 6a. For feedstocks other than lipid extract, the HTL duration had little effect on the HHV of the biocrude samples. The calorific value of the biocrude samples, obtained from a feedstock at different temperatures, were less affected, and no specific pattern was observed; similarly, in another study, there was less difference in HHV values (34.1–35.3 MJ/kg) of biocrude oil obtained from HTL of *Chlorella* sp. at 350 °C [49]. Although the biocrude yield from the carbohydrate extract was the lowest, the HHV of the biocrude samples from carbohydrate extract was similar to that obtained from protein extract. Qiu et al. (2019) reported HHV of biocrude samples from a mixture of leucine and glucose in the range of 26–38 MJ/kg [58]. In this study, the HHVs of biocrude samples-obtained from protein extracts were in the lower range of 27.1–29.6 MJ/kg. Biocrude obtained from carbohydrate-rich feedstock could have cyclic ketones, acids, phenols, and aldehydes that contain oxygenated functional groups in their ringed aromatic structures [19]; higher oxygen content in the biocrude would reduce the HHV. The biocrude samples, derived from microalgal lipid, had the highest HHV for any HTL duration compared to the corresponding values obtained from the biocrude

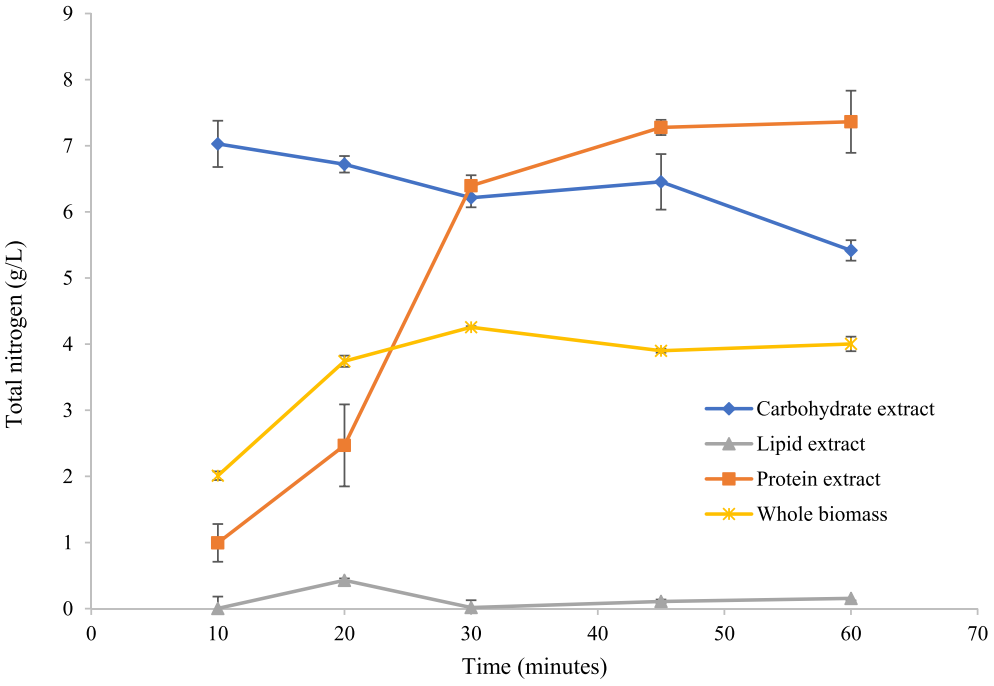

Fig. 5a. Concentration of total nitrogen (TN) in the APL.

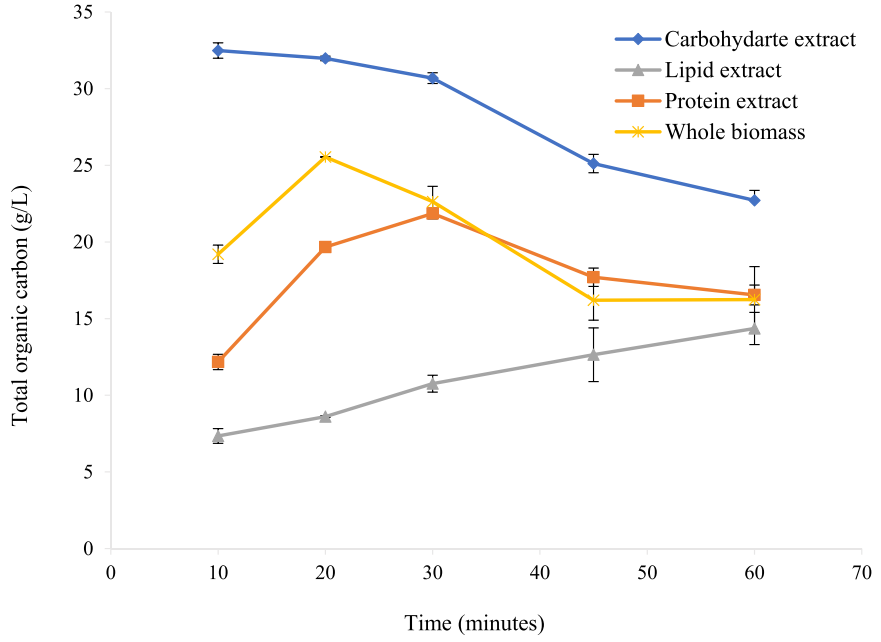

Fig. 5b. Concentration of total organic carbon (TOC) in the APL.

of other macromolecules. It should be mentioned here that the lipid feedstock already had very high HHV (i.e., 37.7 MJ/kg) as compared to the HHVs of other feedstocks. For lipid feedstock, increasing HTL duration over 30 min reduced the biocrude calorific value sharply, which was even lower than that of the original feedstock. The HHV of biocrude samples from whole biomass feedstock was in the range of 30.3–31.6 MJ/kg; in earlier studies, the HHV values for biocrudes from *Chlorella*, *Spirulina,* and *Tetraselmis* sp. were reported in the range of 27.9–34 MJ/kg [59,60].

The maximum energy recovery from whole biomass, lipid extract, protein extract, and carbohydrate extract were 57.7, 84.6, 73, and 44.9%, respectively (Fig. 6b). The HTL duration mainly influenced the biocrude yield, whereas the variation in the HHV values for any feedstock varied in the range of 3.2–20.7%. Therefore,

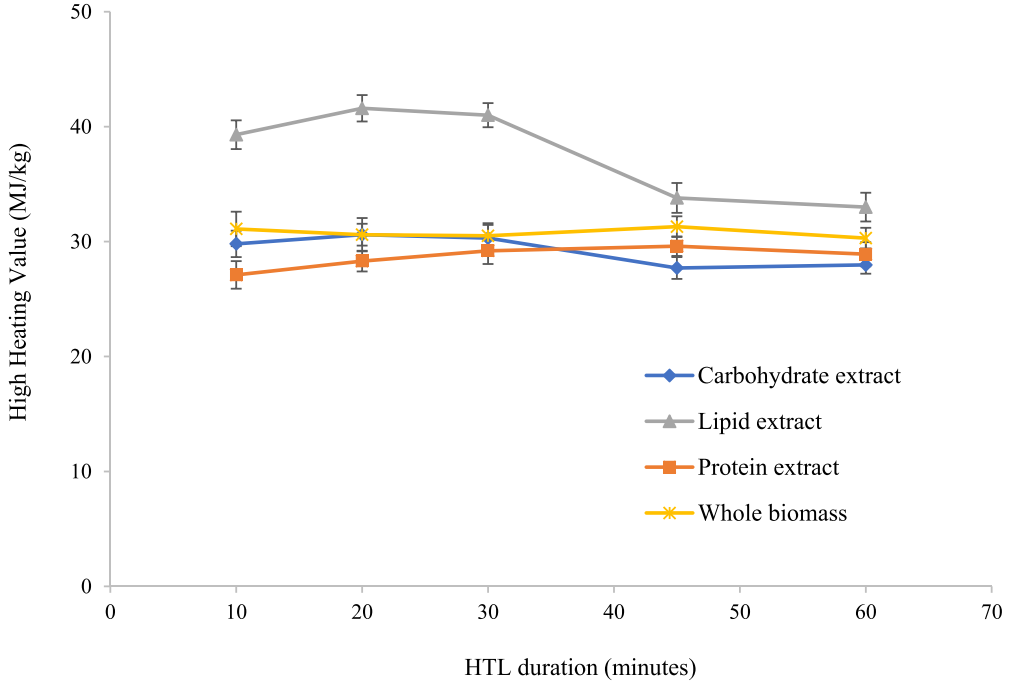

Fig. 6a. HHV of biocrude samples obtained from HTL of different microalgal macromolecules at 350 °C and reaction time of 10–60 min.

the energy recovery value from the feedstock was dominated by the biocrude yield. Although the maximum energy recovery from lipid feedstock was obtained at 20 min, the maximum energy recovery was obtained at 45 min of HTL duration for all other feedstocks. The energy recovery from the microalgal biomass was reported from 38 to 87%, which could be mainly attributed to the feedstock quality and the HTL operating conditions [4,59,61]. Eboibi et al. (2014) reported an increase in energy recovery over increasing HTL duration from *Tetraselmis* sp. biomass at 330 °C; however, at 350 °C, there was a general decline in energy recovery with an

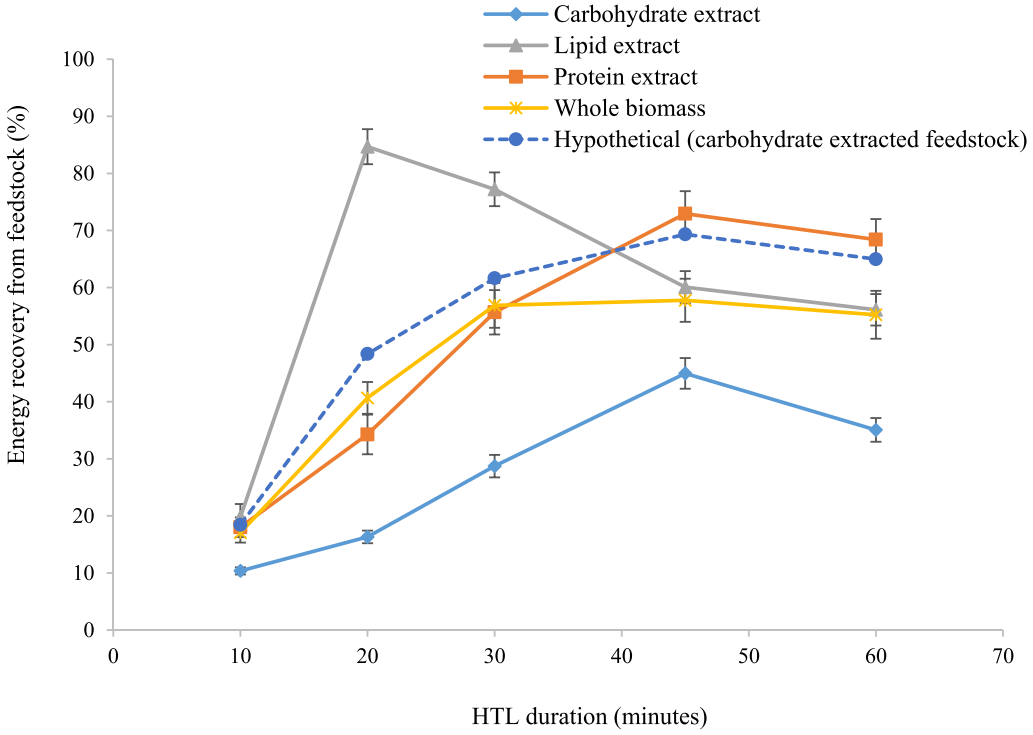

Fig. 6b. Recovery of calorific values in the biocrude oils extracted from the HTL of *Tetraselmis* sp. biomass and its macromolecules extracts at 350 °C.

increase in HTL duration [59]. In this study, at 350 °C, the whole biomass's energy recovery increased with an increase in HTL duration from 10 to 30 min. The preheating and cooling processes and duration were different for this study and the study by Eboibi et al. (2014), which could contribute to the difference in the energy recovery over HTL duration [59].

The energy recovery from carbohydrate feedstock was the lowest, and the loss of carbon in the APL was the highest. Therefore, microalgal biomass with low carbohydrate content could be selected as a feedstock for HTL. Another approach could be the separation of carbohydrate extract in the first phase at a low temperature followed by the second phase at high temperature and pressure; therefore, the separation of carbohydrate would neither require additional chemical nor additional heating energy. The hypothetical energy recovery potential from carbohydrate extracted biomass was higher than the original feedstock for all the HTL duration (Fig. 6b). Furthermore, the extracted carbohydrate from this research work could be used as a feedstock for many other products (lactic acid and bioethanol) through the fermentation process [62,63]. Since 31% nitrogen of the biomass feedstock was liberated with the carbohydrate extract, it could reduce the nitrogen content in the biocrude from the carbohydrate extracted feedstock.

4. Conclusion

This study investigated the effect of HTL temperature and duration on biocrude yield, energy recovery, and products distribution from the macromolecules that were extracted from *Tetraselmis* sp. The biocrude yield from the metabolites extracts followed the order of lipid > protein > carbohydrate for the HTL experiments conducted at 275–350 °C for10 - 60 min. A maximum biocrude oil yield of 77% was obtained from lipid extract for 20 min of HTL at 350 °C. Maximum biocrude yield from protein and carbohydrate extracts were 54 and 22%, respectively, at 350 °C and 45 min of HTL duration. APL generated from carbohydrates had the highest loss of TOC and TN compared to the APL generated from other extracts. HTL duration time could affect the biocrude energy recovery and partitioning of nutrients from the extracted metabolites to the APL. Although maximum energy recovery was from lipid extract was obtained at 20 min of HTL, for other extracts, maximum energy recovery was obtained at 45 min of HTL. Therefore, in a two-phase HTL, microalgal carbohydrate extract could be separated at a lower temperature, followed by the HTL of remaining biomass at an elevated temperature to enhance the energy and nutrient recoveries.

CRediT authorship contribution statement

Hareb Aljabri: Methodology, Writing – original draft, Writing – review & editing. **Probir Das:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing. **Shoyeb Khan:** Data curation, Writing – original draft. **Mohammad AbdulQuadir:** Data curation, Writing – original draft. **Mahmoud Thaher:** Data curation, Writing – original draft. **Alaa H. Hawari:** Writing – review & editing. **Noora Mahmoud Al-Shamary:** Formal analysis, Writing – original draft.

Declaration of competing interest

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

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