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Two-stage hydrothermal liquefaction of a high-protein microalga

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ABSTRACT

Hydrothermal liquefaction (HTL) is a promising route for producing renewable fuels and chemicals from algal biomass. However, the protein fraction of the alga gives rise to nitrogen compounds in the oil fraction, which may render the oil unattractive for use in conventional refining processes. We report a two-stage HTL approach with the primary aim of reducing the nitrogen concentration in the bio-crude oil. A mild (<200 °C) pre-treatment step (Stage I) is performed before more severe (250–350 °C) HTL conditions (Stage II) are applied to the microalga *Chlorella* for the production of bio-crude in a batch reactor. The pre-treatment resulted in up to 50 wt.% of the input nitrogen crossing into the Stage I aqueous phase and, following Stage II processing, reductions in the bio-crude nitrogen contents of up to 55%, relative to the direct HTL of untreated *Chlorella* were observed. However, since considerable amounts of the starting material were lost in Stage I, overall lower quantities of bio-crude were isolated after Stage II processing, as compared to single-stage processing. Nitrogen extraction during Stage I is enhanced by the addition of acids (1 N sulphuric or formic acid) but the process remains unselective. Overall, it is concluded that the two-stage approach to reducing the nitrogen content of bio-crudes from a protein-rich alga requires careful evaluation of the trade-off between the desired bio-crude properties and the yield obtained.

1. Introduction

Hydrothermal liquefaction (HTL) has received increasing interest in the past decade as a process for converting biomass to drop-in biofuels and chemicals [1,2]. The HTL of biomass essentially mimics the natural geological processes believed to be responsible for the formation of fossil fuels in a time frame measured in minutes rather than over a geological time span [3].

Reaction temperatures of approximately 250–350 °C, and pressures high enough to maintain the water as a liquid (i.e., 40–250 bar), are generally employed. The biomass feedstock can be processed directly, without an energy-consuming drying step, since water acts both as solvent and catalyst [4]. The HTL of whole biomass yields a range of different products including bio-crude oil, aqueous dissolved chemicals, solid residue, and gas. The product yields and properties vary significantly according to the feedstocks processed as well as the reaction conditions employed.

Extensive research has been conducted processing lignocellulosic biomass under a range of subcritical water conditions [4,5]. More recently, a wide variety of aquatic plants – including micro [6,7] and macro algae [8] – have also been studied. Processing microalgal feedstocks via HTL possesses numerous advantages over other conversion routes, tolerating low cell concentrations (since HTL is a wet processing method), as well as allowing conversion of low-lipid strains, which often have much higher growth rates than those optimised to accumulate high lipid levels [6].

The algal bio-crude produced by HTL has been described as being similar to conventional crude oil, but bio-crude has significantly higher oxygen and nitrogen contents, typically ~10 to 20 wt.% and ~1 to 8 wt.% respectively [8,9], than conventional crude (both elements <1 wt.% [10]). In particular, processing high-protein algae has been found to significantly increase the nitrogen levels of the derived oils. This can impart a number of undesirable properties, including high viscosity and instability towards cross-linking/oligomerisation and can poison catalysts in conventional refining processes [11].

Bio-crude nitrogen derives from the protein present in the algal feedstock. Increasing HTL temperatures enhance the yield of the bio-crude, while also reducing its oxygen content, but these benefits come at the cost of increasing nitrogen content. This has raised the question as to whether it is possible to extract nitrogen-containing components prior to high-temperature HTL (which is carried out typically at temperatures in the range of 300 to 350 °C). In particular, reductions in nitrogen contents of the final bio-crude product are achieved through an

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initial mild HTL (temperatures < 200 °C) as a preliminary treatment, albeit with concomitant reduction in the quantity of the bio-crude generated [12–15]. While these results are promising, the relationships between quality and quantity of the bio-crude produced in such two-stage treatments are poorly understood. Furthermore, previous work was carried out under slow heating conditions and over extended reaction times, conditions likely to be uneconomical in large-scale production plants. Our previous work on continuous [7] and rapid batch heating [8] has shown that HTL of algal biomass can be achieved at significantly shorter reaction times (<10 min) and it is important to understand the trade-offs under these conditions between reducing nitrogen quality through mild hydrothermal pre-treatment and maximising bio-crude yield. Here we note that protein hydrolysis in hot aqueous environments is generally very slow and that the standard laboratory conditions for complete hydrolysis are strongly acidic (typically 6 N HCl at 110 °C for 24 h) — therefore, we have also investigated the use of acidic media for the preliminary hydrothermal treatment, with a view to enhancing the rapid extraction of insoluble peptides prior to HTL for bio-crude production. In this paper we investigate the relationship between the yield and the nitrogen content of bio-crude when a microalgal biomass is subjected to two-stage HTL in which the first stage is at lower temperatures. We also investigate the effect of carrying out the first-stage HTL under acidic conditions, comparing the results from the addition of an inorganic acid (H_2SO_4) with those obtained with the use of formic acid, representing organic acid that could in principle be produced from biomass.

2. Materials and methods

2.1. Algal biomass

The microalga investigated (*Chlorella vulgaris*, purchased from Synergy Natural Limited) was grown in freshwater ponds before spray drying and packaging in powder form. The biochemical composition (lipids, protein, carbohydrates) was provided by the supplier. Moisture content was determined by weight loss from a ~3 g sample upon heating in an oven at 105 °C for 2 h while the ash content was determined from the residual mass obtained after heating ~3 g of dried sample in a muffle furnace for 3 h at 550 °C. The elemental analysis (C, H, N and S, with O determined by difference) was obtained using a commercial analyser (Flash EA 1112, CE Instruments, UK). The proximate and elemental analyses are reported in Table 1 as the average of duplicate assays, the maximum deviation between runs being ±0.3%. These analyses are typical of a low-lipid, high-protein microalga.

Table 1
Analysis of *Chlorella* feedstock.

Analyses	*Chlorella*
Proximate (wt.% as received)	
Ash	6.0
Moisture	5.2
Elemental (wt.% daf)[a]	
C	53.5
H	7.4
N	11.0
S	0.5
O[b]	27.6
Biochemical (wt.% as received)	
Carbohydrates	25
Protein	60
Lipids	4

daf = dry ash free.
[a] Average of duplicates; variation < 0.3%.
[b] By difference.

2.2. Reactor setup

Reactions were carried out in a small-scale (20 mL) rapid-heating/cooling batch reactor system described previously [8]. Briefly, the reaction volume is formed by a 120 mm length of ¾″ outer diameter stainless steel 316 tube (wall thickness 1.65 mm), closed at one end and connected to gas supplies via a length of ¼″ tubing. Swagelok fittings are used throughout, enabling rapid dismantling and reassembly of the system. The reactor is loaded with biomass/water (see below) and purged with nitrogen before being pressurised, also with nitrogen, to ~90 bar. It is then immersed in a pre-heated fluidised sand bath (Techne SBL-2D) to raise the temperature to the desired experimental set-point. The temperature in the reactor is measured through a centrally located K-type thermocouple — typically, the internal temperature approached within <10 °C of the set-point within 1–2 min. The reaction times reported include these heat-up periods. Transport of water vapour out of the reactor volume is inhibited by restricting the opening in the connecting tube to an annular opening of 0.165 mm over a 50 mm length of the ¼″ connecting tubing. At the completion of the reaction period the reactor volume, still attached to the connecting tubing, is plunged into an ice bath, causing the temperature to decrease to <20 °C within 30 s.

2.3. Processing methodology and analytical techniques

Fig. 1 describes the two-stage experimental protocol and nomenclature used in the following descriptions. In all cases, the feed to the first stage is a suspension of nominally 20 wt.% dry *Chlorella*, prepared by mixing ca. 2.5 g of dry biomass with 10 mL of aqueous solvent. The feed to the second stage is then made up as a 10% aqueous suspension of the dry solids produced in the first stage. Bio-crude is produced only in the second stage; the first stage did not produce any identifiable oil phase (<1 wt.% yield, as determined by dichloromethane (DCM) extraction, see below). Gas production in Stage I is also expected to be negligible [16].

First stage treatments were performed using three different solvents (distilled water, 5 wt.% sulphuric acid, and 5 wt.% formic acid — the acids are each approximately 1 N) at temperatures in the range of 100–200 °C and at residence times of 5 and 15 min. The product mixture was subsequently collected and centrifuged to separate the Stage I Solid and Aqueous Phase.

The Stage I Solids fraction was dried, weighed and analysed for its elemental composition and ash content. Solid yields were then calculated based on the recovered weight and are reported as a fraction of the dry feedstock. The experiments were conducted in duplicate, for which the maximum absolute error for the solid yield was <2%; only average values are reported.

The Stage I Aqueous Phase was analysed for total organic and inorganic carbon (TOC and TIC, IL550 TOC, Hach-Lange, Germany). The analyses were carried out in duplicate, for which the maximum relative deviation was <2%, and we report the average result. Total nitrogen (TN) was determined by colourimetry (test cuvettes LCK338, Hach-Lange, Germany).

The treated *Chlorella* solids obtained from Stage I were dried, mixed with distilled water, and returned to the reactor for the second stage of the process. All the runs in Stage II were processed with a 10 wt.% feed concentration (1.0 g dry solids with 9 mL distilled water). Reactions were carried out over a range of temperatures (250–350 °C) with a constant reaction time (10 min) and the product yields and quality were determined.

The Stage II HTL oil–water–solid product mixture was decanted and the reactor washed using DCM and distilled water (30 mL each in 15 mL aliquots). The resulting mixture was separated in a separating funnel followed by filtration and DCM solvent evaporation to afford a bio-crude oil, a solid residue and an aqueous phase — as described

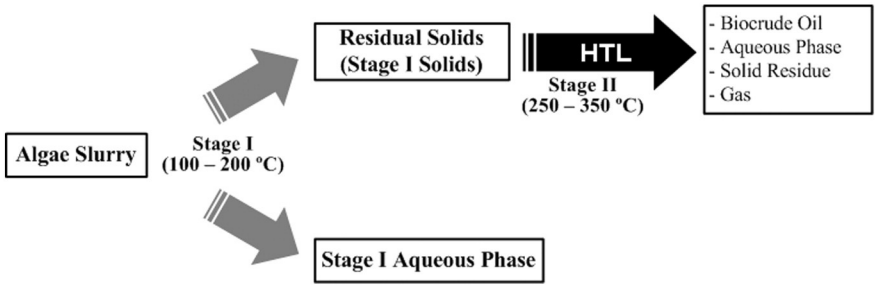

Fig. 1. Schematic diagram of the two-step hydrothermal liquefaction process.

previously [7,17]. The gases were not collected, but vented inside a fume hood as the reactor was opened after the experiment.

The elemental and proximate analyses of the bio-crude oil were obtained as described above. The boiling point distribution of the oil was determined using simulated distillation thermal gravimetric analysis (sim-dis TGA, DTA-673, Stanton Redcroft, UK): 5–10 mg of sample was heated in 50 mL/min of nitrogen to 900 °C at 10 °C/min.

3. Results and discussion

3.1. Stage I: algae pre-treatment

As depicted in Fig. 2 (a, b), yields of Stage I Solids decrease with increasing pre-treatment temperatures for all solvents investigated. At 100 °C, there is no apparent effect of increasing the reaction time and there is no difference between the yields for water and sulphuric acid treatment while formic acid treatment gives slightly higher yields. At higher temperatures, the yield is reduced at the longer time. Within these trends, the effect of acid on the conversion of the initial biomass is mixed — for t = 5 min, the acids reduce the Solids yield when T ≥ 150 °C but for t = 15 min, the yields are all similar at 200 °C.

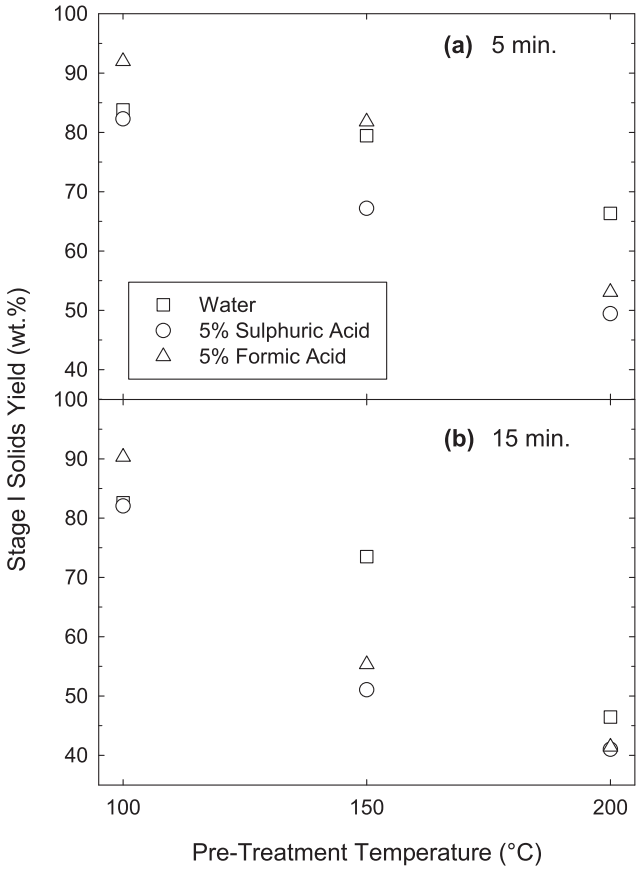

Fig. 2. Effects of different pre-treatment solvents and temperatures on Stage I treated algae yields for reaction times of (a) 5 min and (b) 15 min. Yields are an average of at least two duplicates; maximum absolute error is 2%.

The relatively large yields of solids shown in Fig. 2 correspond to scanning electron microscopy (SEM) observations that these mild HTL treatments do not lead to extensive cell disruption. SEM images of treated and untreated algae solids are included in the electronic supplementary material (Fig. S1). Untreated *Chlorella* cells present as clusters with a size ~50 μm, Fig S1 (a). Some mild agglomeration is apparent after treatment at 100 °C, but there are no significant morphological differences arising as a result of the different treatment times or the use of different solvents, Fig. S1 (b–g). Increasing the temperature to 150 °C results in a greater degree of agglomeration of the residual cells, Fig. S1 (h–m), which themselves remain relatively unchanged (except for some non-uniformity in the surfaces after sulphuric acid treatment). At the highest temperature of 200 °C, Fig. S1 (n–s), while the agglomerated solid structures become more compact, the individual cell units remain recognisable at high magnification for all solvents.

The present results are broadly similar to those reported by others for mild hydrothermal treatment of microalgae in water [10,12] and organic acids [16]. Overall, it is apparent that more severe treatment leads to a significant reduction in the yield of Stage I solids available for the Stage II HTL step. In order to understand the implications of this effect, we need therefore to consider in more detail the changes occurring, which we do here in terms of behaviour of the elemental carbon and nitrogen.

Table 2 summarises the results of the elemental analyses of the feedstock and Stage I Solids as well as the fractions of feedstock that report to the Stage I Solids Phase. From these data, the elemental mass-balance closures (total mass of element in solids and aqueous phase products / mass of element in feedstock) for carbon and nitrogen can be calculated, as summarised in Fig. 3. Overall, the results are satisfactory with means (±1 standard deviations) 94 (±5)% and 87 (±8)%, respectively, but there is an apparent tendency towards a greater deficit when the Stage I Solids recovery is reduced (more extraction, nominally to the liquid phase, especially for nitrogen). For the purposes of the following discussion, we base the deportment of the elements only on the measurements of the mass and elemental compositions of the solids (biomass feedstock and Stage I Solids), thereby eliminating the uncertainty in the aqueous-phase compositions and quantities.

From Table 2, it is apparent that treatment in water produces solids with a nitrogen fraction that exceeds that of the feedstock for all but the most severe conditions (15 min reaction time at 200 °C). However, the N/C ratio remains approximately constant, N/C ~ 0.20 wt/wt, for all conditions except the most severe when it falls to 0.17. It is clear that, even under these relatively mild conditions, the preferential loss of oxygen (O/C decreasing from 0.51 to 0.36 wt/wt over the range of exposures) underlies these observations. This is made clear in Fig. 4 which shows the calculated N/O ratio of the material lost from the feedstock (and reporting to the aqueous phase) for the 15-min reaction times at the different treatment temperatures. At 100 °C, the material extracted from the feedstock has N/O < 0.1 which is much less than the feedstock

Table 2
Elemental composition (wt.% daf) and yield (wt.%) of Stage I Solids.

			C	H	N	S	O[a]	Ash	N/C	O/C	Solid yield
Untreated algae			54.0	7.5	10.6	0.4	27.5	6.0	0.20	0.51	–
Pre-treated algae											
Solvent	*Temp. (°C)*	*Time (min)*									
Water	100	5	54.0	7.7	11.5	0.5	26.3	3.4	0.21	0.49	83.8%
	150	5	54.6	7.9	11.4	0.5	25.6	4.6	0.21	0.47	79.4%
	200	5	57.9	8.2	11.5	0.4	22.0	3.8	0.20	0.38	66.3%
	100	15	58.4	8.3	11.9	0.5	20.9	4.5	0.20	0.36	82.6%
	150	15	59.0	8.3	11.8	0.5	20.4	2.7	0.20	0.35	73.5%
	200	15	59.4	8.3	10.2	0.5	21.6	3.0	0.17	0.36	46.5%
5 wt.% sulphuric acid	100	5	50.7	7.4	10.2	2.1	29.6	[b]	0.20	0.58	82.3%
	150	5	51.5	7.2	9.6	3.4	28.3	2.6	0.19	0.55	67.2%
	200	5	54.0	7.7	11.5	0.5	26.3	3.8	0.21	0.49	49.4%
	100	15	51.7	7.3	9.8	2.5	28.7	3.7	0.19	0.56	82.0%
	150	15	51.6	7.0	8.1	3.4	29.9	2.9	0.16	0.58	51.0%
	200	15	60.8	7.7	6.9	2.6	22.0	4.0	0.11	0.36	41.0%
5 wt.% formic acid	100	5	53.0	7.2	10.1	0.6	29.1	3.3	0.19	0.55	92.0%
	150	5	55.3	7.6	10.6	0.5	26.0	0.6	0.19	0.47	81.9%
	200	5	57.8	7.6	9.0	0.5	25.1	2.8	0.16	0.43	53.1%
	100	15	54.1	7.3	8.8	0.4	29.4	1.7	0.16	0.54	90.3%
	150	15	51.0	8.0	9.2	0.5	31.3	3.4	0.18	0.61	55.3%
	200	15	58.6	7.0	7.9	0.4	26.1	[b]	0.13	0.45	41.4%

daf = dry ash free; proximate and elemental analyses tested in duplicate for each sample and the maximum deviation = ±0.3.
[a] By difference.
[b] Not analysed.

value of N/O = 0.39 suggesting that, at this temperature, the protein content of the feedstock remains mostly in the solid phase and the mass loss is due to solubilisation of oxygen-rich material — in fact, the extract has an O/C ratio of ~0.7, suggesting that sugars and small acids from the hydrolysis of the carbohydrates are preferentially extracted into the solvent. As the temperature is increased, the extraction of nitrogen is enhanced and at 200 °C, the extracted material has N/O = 0.34, approaching that of the feedstock.

Fig. 5 shows the extents of extraction of C and N to the aqueous phase, as measured. For treatment in water, these extents follow very similar trends as the reaction temperature and the reaction time is varied, consistent with the small changes in the C/N ratio of the Stage I Residue. The maximum extent of extraction of nitrogen into the aqueous phase is 36% but this is accompanied by a carbon loss from the solids

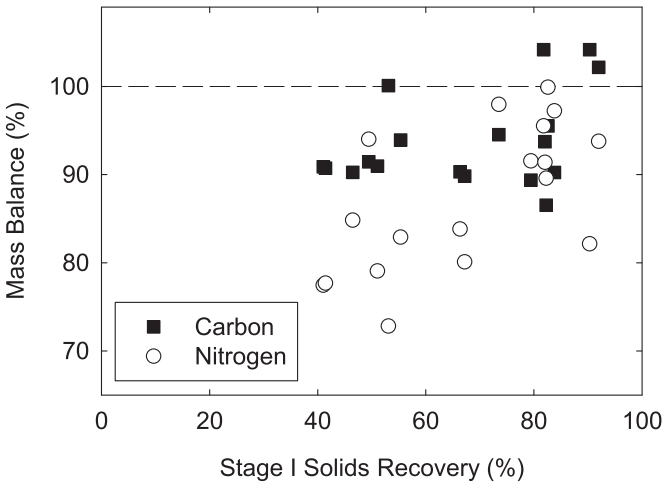

Fig. 3. Stage I mass balance closures for elemental carbon and nitrogen, determined as measured product amounts relative to feed amounts for all experiments. Results are shown as averages of 2 experiments with duplicate elemental analyses; maximum relative error is 5%.

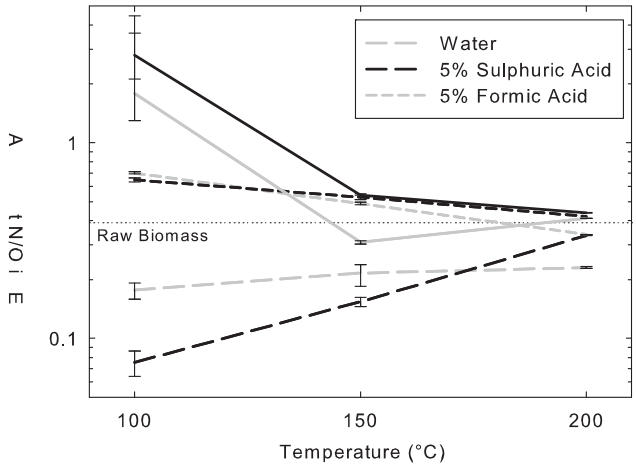

Fig. 4. Calculated mass ratio of N/O in material lost from the raw biomass during Stage I treatment. Black lines are for treatment times of 15 min; grey lines are for 5 min. Error bars show range of variation for 2 experiments with duplicate elemental analyses.

of 34%. Clearly the Stage I treatment in water is quite unselective and only achieves significant removal of nitrogen under relatively severe conditions, which also solubilise carbon.

The behaviour of vegetable protein under mild hydrothermal conditions has been reported in the literature. Pińkowska and Oliveros [18] found negligible extraction of soybean protein after 30 min at temperatures below 140 °C; above this temperature, extraction was much more efficient, leading to maximal yields of ~60% at temperatures of around 250 °C. Yu et al. [19] reported that nitrogen extraction from *Chlorella pyrenoidosa* only becomes significant at temperatures above 160 °C (reaction time 30 min); at 200 °C, the retention of nitrogen in the solid mirrors that of carbon at short reaction times. Qualitatively, these observations are in agreement with those made here.

More fundamental studies of protein reactions under HTL conditions show that thermal protein denaturation and insolubilisation occur in parallel with solubilisation by peptide hydrolysis — hydrolysis is slow in water but is greatly accelerated by acids [20]. Since organic acids are readily formed from the hydrolysis of carbohydrates, we suggest that acid-catalysed peptide hydrolysis is responsible for increasing extraction of nitrogen into the aqueous phase at higher temperatures and longer reaction times. The ultimate extent of extraction of protein nitrogen under more extreme conditions may be limited by insolubilisation and the fact that hydrolysis products themselves are liable to repolymerise at higher temperatures [20].

By carrying out the Stage 1 treatment in acid, it is expected that protein hydrolysis will be enhanced, especially under milder conditions when the formation of organic acids is retarded. Indeed, while acid extraction has apparently modest effect overall on the yield of solid residue (Fig. 2), it is clear from Fig. 4 that the differences in the extract composition under milder conditions are profound — the calculated N/O ratios of the acid extracts at 100 °C increase at least 10-fold relative to water extraction. This change is clearly due to a greater extent of nitrogen extraction which occurs more or less congruently with carbon extraction (Fig. 5) so that the decline in the N/C ratio of the solid residue remains modest (Table 2). At 150 °C, the extraction of N and C into the aqueous phase (15 min reaction time) is further enhanced in the presence of acid while extraction into water remains slight. However, there is little further extraction into acid at 200 °C, whereas extraction into water increases substantially, to the point where there is little difference between the results for the longer reaction time.

The fate of inorganic components in Stage I treatment can also be ascertained from Table 2. The ash/C ratio in the raw biomass is 0.11 and becomes 0.06 ± 0.02 (1 s.d.) regardless of processing conditions — it appears that the inorganic (ash) removed during Stage I treatment is

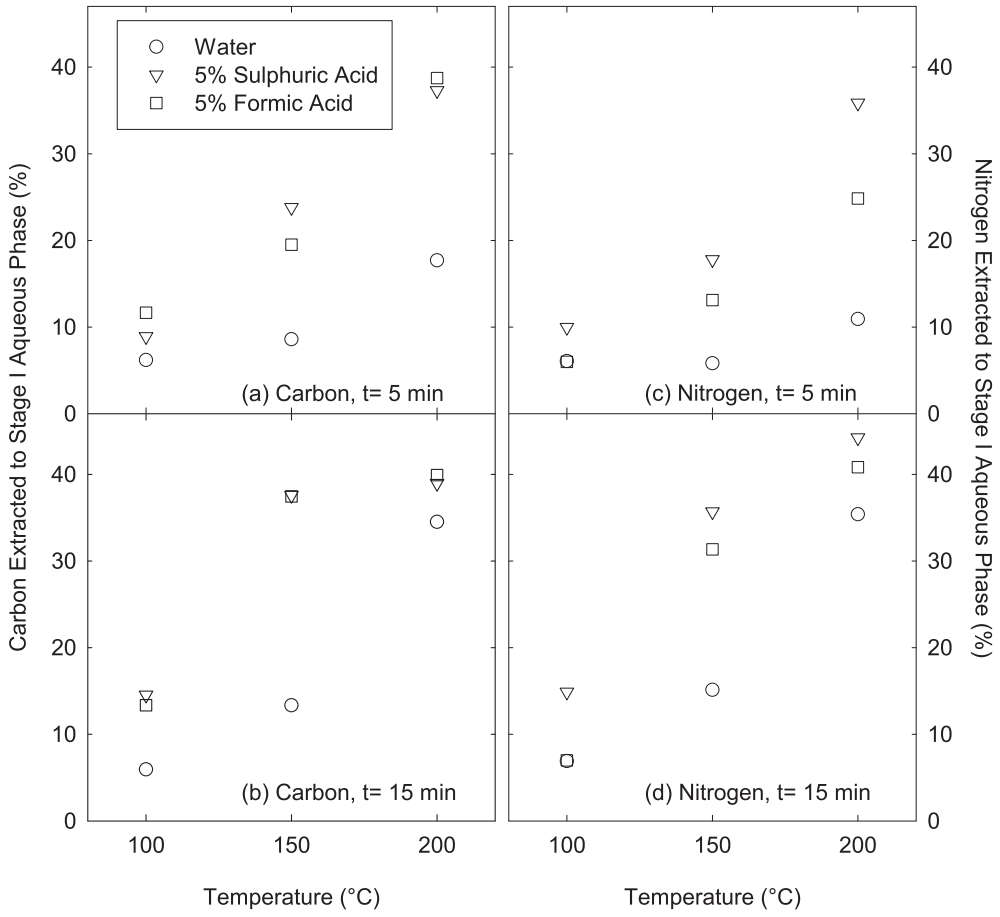

Fig. 5. Stage I aqueous phase recoveries for carbon, panels (a) and (b), and nitrogen, panels (c) and (d). Upper panels are for 5 min reaction time; lower panels are for 15 min reaction time. Results are shown as averages of 2 experiments with duplicate elemental analyses; maximum relative error is 10%.

readily water-soluble, with the residual ash being inert under the range of conditions studied here. Here we note that the appearance of additional sulphur in the Stage I Residue after treatment in sulphuric acid suggests uptake of some sulphate in the amount sulphate/C ~ 0.10 which clearly exceeds the measured ash/C, suggesting that the added sulphur is not ash-forming and may be organically bound.

3.2. Stage II: hydrothermal liquefaction

The residual solids obtained from Stage I were hydrothermally processed at a range of temperatures (250–350 °C) for a fixed reaction time (10 min), in order to evaluate the product yields and quality. The concentration of dry Stage I solids fed into the second stage was maintained at 10 wt.% for all runs.

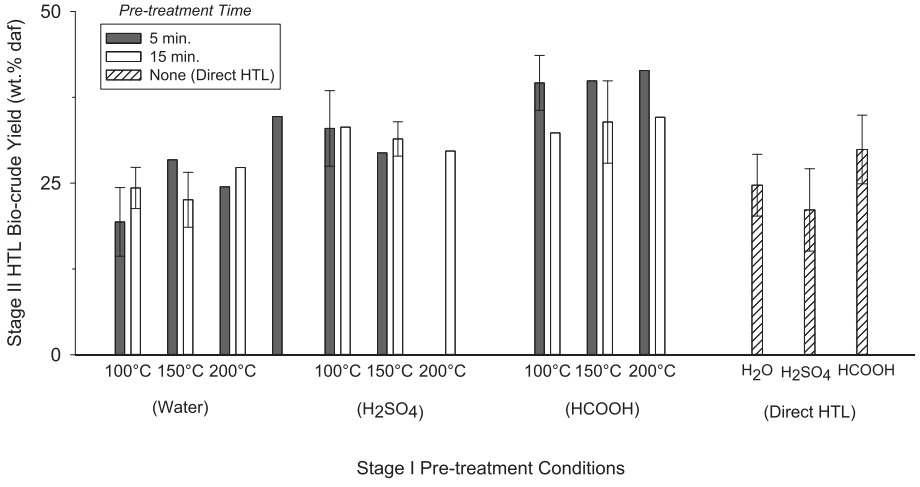

Fig. 6. Stage II HTL bio-crude yields obtained at 300 °C, 10 min reaction time and 10 wt.% solids loadings for algae pre-treated at various conditions. Error bars are an average of at least two repeats; maximum absolute error is 5%.

Fig. 6 shows bio-crude yields for Stage II HTL at 300 °C where the stage-wise yield is defined in terms of the fraction of the mass of Stage I residual solids being processed. For each of the three Stage I solvents, variations in pre-treatment time or temperature have little effect on the conversion of the solids to bio-crude — only formic acid treatment shows a consistent effect, a slightly reduced yield after the longer (15 min) pre-treatment at all pre-treatment temperatures. Compared with direct HTL of raw *Chlorella* (without pre-treatment), the yields after water pre-treatment are unchanged, ~25% by weight; pre-treatment with sulphuric acid yields ~32% oil (compared with 21% for direct HTL in the acid medium) while formic acid pre-treatment gives rise to a yield of 40% (30% for direct HTL in the acid). Clearly, acid pre-treatment, even at 100 °C, produces solids that are more efficiently converted to bio-crude than the solids remaining after water pre-treatment. Surprisingly, direct HTL of the raw algae in either acid medium at 300 °C has a lower yield than the acid-treated algae. Direct HTL in the acid media produces amounts of bio-crude approximately similar to direct HTL in water (25 ± 5%).

The overall bio-crude yield from the two-step hydrothermal liquefaction process depends not only on the efficiency of converting the Stage I solids to bio-crude, but also on the fraction of the original biomass reporting as such solids. Fig. 7 presents these overall yields (for a 15 min Stage I treatment) and compares them with the results for direct HTL. It is apparent that pre-treatment in water, even at 100 °C, leads to a reduction in the overall bio-crude yield and that this loss is magnified at higher pre-treatment temperatures. For the acid pre-treatments, while there may be a slight improvement in overall yield after pre-treatment at 100 °C, the general observation holds true, that the Stage II yield enhancement fails to offset the loss of solids incurred in the pre-treatment. Indeed, the results with the different solvents are indistinguishable at pre-treatment temperatures of 150 and 200 °C.

As shown in Fig. 8(a) for HTL after Stage I treatment for 15 min at 150 °C, the effect of increasing the HTL temperature is generally to enhance the stage-wise yield. For water, these yields are essentially the same as those obtained in direct HTL of the raw biomass, suggesting that the solids obtained after the first stage treatment behave much the same as untreated material in their propensity to produce bio-crude. However, as shown in Fig. 8(b), the overall yield is about 25% lower after pre-treatment, reflecting the extraction of solids during Stage I.

The effects of HTL temperature on acid pre-treated samples are more complex. At 250 °C, the bio-crude yields after acid pre-treatment

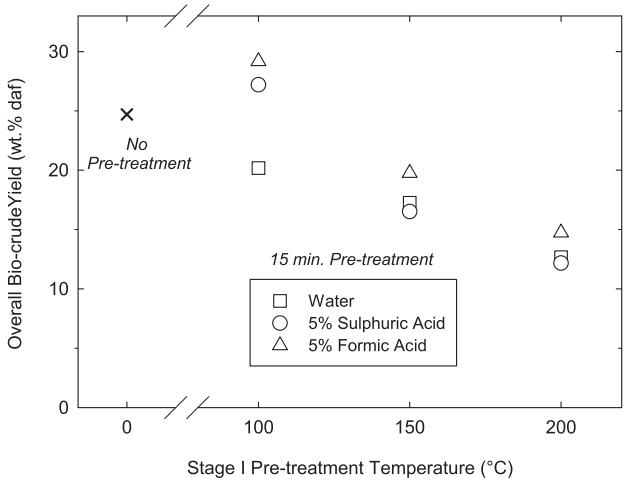

Fig. 7. Overall bio-crude yields for Stage II HTL at 300 °C, 10 min reaction time with 10 wt.% biomass loadings after Stage I algae treatments carried out between 100–200 °C for 15 min. Also shown at the left are the yields obtained from direct HTL of untreated algae. Results are shown as averages of 2 experiments with duplicate analyses; maximum relative error is 10%.

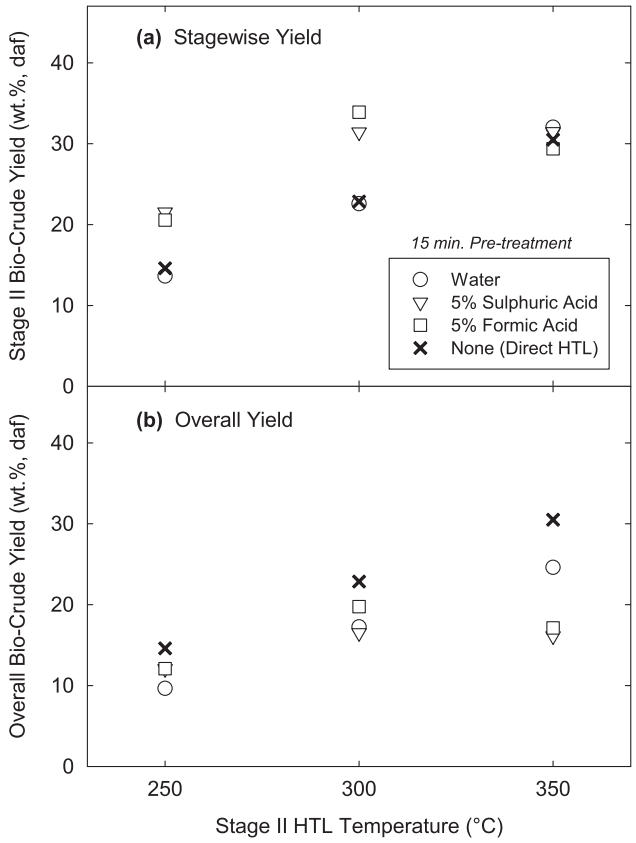

Fig. 8. HTL yields for a range of temperatures with 10 min reaction time and 10 wt.% biomass loadings. Stage I algae treatments carried out at 150 °C for 15 min. Yields calculated on the basis of solids fed into (a) Stage II and (b) Stage I. Results are shown as averages of 2 experiments with duplicate analyses; maximum relative error is 10%.

(20–22 wt.%) are significantly greater than those from direct HTL (~14 wt.%). However, allowing for the reduction in solids available for HTL as a result of the pre-treatments at 150 °C, the overall bio-crude yield after HTL is actually slightly less than is obtained from direct HTL. Therefore, while the acid pre-treatments have had a profound effect on the nature and quantity of the solids going into the HTL stage, the overall impact on bio-crude yields is muted. Although the Stage II bio-crude yield at 300 °C is substantially greater than at 250 °C, the same overall behaviour is apparent. On the other hand, the yield from Stage II HTL at 350 °C is actually lower than at 300 °C, with the result that the overall bio-crude yield is approximately half that for direct HTL.

In their study of a two-step sequential hydrothermal process, Miao et al. [12] achieved greater overall bio-crude yields from treated microalgae (*Chlorella sorokiniana* pre-treated at 160 °C for 20 min in water; Stage II HTL at 220–260 °C and 60 min reaction time), compared to a single step HTL process. This result requires that the biomass fed into the second stage yields at least 60% bio-crude, which is far greater than any yields we observed (with shorter pre-treatment times and substantially shorter HTL reaction times). The differences may relate to the different HTL reaction times or to the different nitrogen contents (2.9% versus 10.6% N in the present study).

Elemental analyses of the various bio-crudes obtained in this work are shown in Table 3. Overall, it is apparent that the effect of pre-treatment is to reduce the nitrogen content of the bio-crude, as is shown graphically in Fig. 9 for HTL at 300 °C and 10 min reaction time after pre-treatment at 150 °C for 15 min. The lowest nitrogen content (3.4%) is obtained from solids pre-treated in water for 15 min at 200 °C; under these conditions, the shorter pre-treatment time (5 min) gives rise to a bio-crude nitrogen concentration of 4.5% (Table 3). Acid pre-treatments, especially with formic acid, are accompanied by higher

Table 3
Elemental composition (wt.% daf) of bio-crude derived from the direct HTL of *Chlorella* and two-step liquefaction process under various conditions. The bio-crude yield in the right-most column is calculated as fraction of the Stage I Solids that is converted to bio-crude, on a daf basis.

					C	H	N	S	O[a]	Bio-crude yield
Direct HTL			*HTL conditions*							
Solvent			*Temp. (°C)*	*Time (min)*						
Water			250	10	74.7	10.4	4.1	0.1	10.7	14.6%
			300	10	73.1	9.3	7.5	0.3	10.3	24.7%
			350	10	73.2	9.5	7.4	0.1	9.8	30.5%
Stage I pre-treatment conditions			*Stage II HTL conditions*							
Solvent	*Temp. (°C)*	*Time (min)*	*Temp. (°C)*	*Time (min)*						
Water	100	5	300	10	71.2	8.7	6.6	0.4	13.1	19.4%
	150	5	300	10	70.8	8.5	6.1	0.4	14.2	28.4%
	200	5	300	10	70.2	8.1	4.5	0.4	16.8	24.5%
	100	15	300	10	70.9	9.0	6.1	0.5	13.5	24.3%
	150	15	250	10	71.5	9.6	4.5	0.2	14.2	13.6%
	150	15	300	10	71.0	8.8	4.2	0.4	15.6	22.6%
	150	15	350	10	70.7	9.1	6.4	0.3	12.7	32.0%
	200	15	300	10	73.2	9.8	3.4	[b]	11.2	27.3%
5 wt.% sulphuric acid	100	15	300	10	70.8	9.0	5.3	0.4	14.5	33.2%
	150	15	250	10	74.5	9.4	5.0	0.3	8.4	21.5%
	150	15	300	10	73.3	9.4	4.7	0.3	12.3	31.4%
	150	15	350	10	75.9	9.7	4.8	0.6	8.7	31.5%
	200	15	300	10	75.8	9.8	4.0	0.3	10.1	29.7%
5 wt.% formic acid	100	15	300	10	72.9	9.2	6.8	0.2	10.9	32.3%
	150	15	250	10	74.3	10.4	3.6	0.2	11.5	20.6%
	150	15	300	10	73.4	9.6	6.7	0.3	10.5	33.9%
	150	15	350	10	73.5	10.3	6.4	0.3	9.5	29.4%
	200	15	300	10	75.2	9.7	5.9	0.2	9.0	34.6%

daf = dry ash free; elemental analysis tested in duplicate and the maximum deviation = ±0.3.
[a] By difference.
[b] Not analysed.

nitrogen concentrations, even though the Stage I solids have slightly lower N/C nitrogen after the acid treatments (Table 2 and Fig. 5 (d)). Clearly, the N/C ratio of the pre-treated material is not a good guide to the nitrogen content of the oil obtained after HTL. The picture is complicated further by the fact that the relative effectiveness of the different pre-treatments changes with different HTL temperatures (Table 3). Notwithstanding the complexities in these results, it is apparent that acid pre-treatment, while greatly enhancing the extraction of protein under mild pre-treatment conditions, does not produce any significant advantage over water pre-treatment. Even with water pre-treatment, any significant reduction in nitrogen content of the bio-crudes is achieved at the cost of a substantial loss in overall oil yield (Fig. 8b) — evaluating the trade-offs between these effects is beyond the scope of this investigation.

The bio-crudes in the current study were also analysed for their carbon, hydrogen and sulphur contents, with oxygen being determined by difference, see Table 3. Algae subjected to different pre-treatment conditions produce bio-crudes in the subsequent HTL step with similar carbon (69–76 wt.%), hydrogen (8–10 wt.%), sulphur (0.2–0.5 wt.%) and oxygen (9–15 wt.%) contents. Some specific differences are noteworthy: bio-crude produced after Stage I treatment in sulphuric acid shows no elevation in sulphur content even though the Stage I solids contained up to 4% sulphur, presumably in the form of organic sulphates. Additionally, the oxygen contents of the bio-crudes obtained after pre-treatment in water are higher than those from HTL of the raw or acid-treated biomass — although the oxygen content values are obtained by difference, the effect appears to be systematic.

Raising the HTL reaction temperature has little effect on the carbon and hydrogen contents of the bio-crude fraction, while the oxygen levels are slightly reduced; this is the case for bio-crudes derived from both treated and untreated algae, in agreement with the findings from [7] for the continuous HTL of untreated *Chlorella* as well as results published by Miao et al. [12] and Jena et al. [21]. For HTL at temperatures > 250 °C, the bio-crude yields generally increase with increasing temperature (Fig. 8), so the fraction of the original biomass carbon reporting to the bio-crude also follows this trend, as reported by Yu et al. [19].

The boiling point distributions of the bio-crudes obtained from HTL at 300 °C and 10 min reaction time, determined through simulated distillation by TGA, are presented in Fig. S2. Similar results are obtained for bio-crudes derived from untreated and pre-treated algae (Stage I treatments carried out for 15 min in various solvents at 100 and 200 °C), falling predominantly in the distillation range of Kerosene. Stage I solids from pre-treatment with sulphuric acid, however, produce bio-crude with an enhanced Kerosene distillate fraction and a reduced Heavy Naphtha fraction. Additionally, higher pre-treatment temperatures have a similar effect, producing a slightly greater Kerosene fraction while reducing the Heavy Naphtha fraction.

The bio-crudes obtained in [7] from the continuous HTL of *Chlorella* presented more uniform boiling point distributions than those from the two-step as well as the direct batch HTL processes. This is attributed to the shorter reaction times used in the continuous process which resulted in greater yields of higher molecular weight fractions. The data presented in Fig. S2 are more closely correlated to the results of Biller et al. [22] who found greater yields of lower molecular weight bio-crude fractions while processing untreated *Chlorella* at 350 °C for 60 min in a batch reactor.

4. Conclusions

Pre-treatment of microalgae under mild hydrothermal conditions solubilises some of the raw biomass, leaving a residue that produces bio-crude under more severe (higher-temperature HTL) conditions. In particular, the extraction of nitrogen in the pre-treatment stage allows some reduction in the nitrogen content of the HTL bio-crude product. However, the nitrogen-containing (protein) component of the biomass is resistant to solubilisation under mild hydrothermal conditions and it is only at higher pre-treatment temperatures (>~150 °C) that significant nitrogen extraction is achieved in reasonable reaction periods. Under these conditions, there is significant concomitant solubilisation of

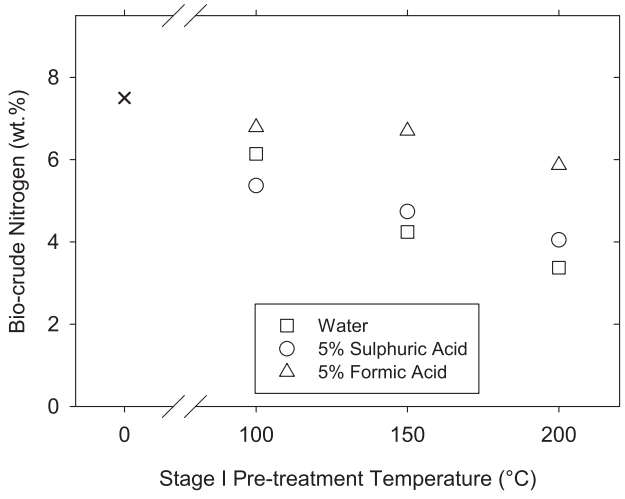

Fig. 9. Nitrogen content of bio-crudes obtained from Stage II HTL at 300 °C and 10 min. Stage I algae treatment time = 15 min. Results are for average of 1 to 4 repeat analyses; relative standard deviation < 5%.

carbon. For the protein-rich *Chlorella* studied here, the nitrogen extraction step is therefore unselective, with the result that the N/C ratio of the solid residue remains relatively constant even when more than 50% of the original biomass has been extracted. While the N/C ratio of the residue is not the only determinant of the nitrogen content of bio-crude produced in the subsequent HTL stage, the overall reduction in nitrogen content of the bio-crudes obtained is limited.

The solubilisation of protein under mild conditions, ~100 °C, is enhanced in the presence of acids, presumably through acid hydrolysis. However, higher temperatures (>~150 °C) are still required in order to achieve significant levels of nitrogen extraction in reasonable times, under which conditions the differences between pre-treatment in water and in 1 N sulphuric or formic acids are less pronounced, possibly because autogenous acid production becomes sufficient to induce protein hydrolysis to an extent similar to that induced by the added acid.

In this work, the bio-crude obtained by direct HTL of the raw biomass at 300 °C is 7.5% (daf) nitrogen, while pre-treatment at 200 °C enables this value to be reduced to 3.4%. This result must be considered in the light of the fact that the overall bio-crude yield decreases from 24.7% to 12.7% for the corresponding direct and two-stage HTL processes. For this high-protein alga, the pre-treatment step is essentially unselective and the economic benefits of achieving lower nitrogen content in the bio-crude may be outweighed by the cost of providing an additional processing step and its concomitant reduction in overall yield. Addition of acids to promote protein hydrolysis does not appear to offer significant benefits overall.

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Appendix A. Supplementary data

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