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Subcritical water extraction of lipids from wet algae for biodiesel production

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HIGHLIGHTS

- Extraction of lipids/oil from wet algal biomass.
- Water was used as solvent for extraction.
- Complete extraction of lipids using microwave assisted heating.
- 2–8 Times reduction in extraction energy.
- Observed potential byproducts (proteins, omega-3 fatty acids, sugars).

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ABSTRACT

An energy efficient extraction of algal lipids from wet algal biomass was performed at subcritical conditions of water. This was achieved using microwave assisted heating as well as conventional heating. The conventional heating subcritical water (C-SCW) extraction and microwave assisted subcritical water (MW-SCW) experiments were designed and conducted to study the effects of extraction temperature, time, and biomass loading on lipid extraction. The Response surface methodology was used to optimize the parameters for maximum extraction of lipids. The influence of extraction temperature is more when compared to other experimental parameters in both processes. The maximum extraction efficiencies were achieved at 220 °C using conventional heating and 205 °C using microwave heating. Complete extraction of lipids was observed with microwave assisted heating and 70% extraction efficiency was achieved using conventional heating. The energy required for extraction is greatly reduced (2–8 folds) when compared to the conventional solvent extraction. The potential by-products like protein rich residual algae, omega-3 fatty acids, and sugars in residual water phase were identified. The biomass and the crude extracts were characterized using GC–MS, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), and thermogravimetric analysis (TGA).

1. Introduction

The need for alternative energy sources to replace fossil fuels has motivated many researchers and policymakers to develop innovative research programs around the world. Development of biofuels for the transportation sector is one of those programs directed towards production of sustainable renewable fuels and significant progress has been achieved in development of some renewable biofuels. Biodiesel is best known among renewable fuels and is currently being produced from a wide variety of vegetable and plant oils. The implication of the utilization of vegetable oils to produce biodiesel has increased demand on the domestic markets and in some instances the production is often reduced

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due to scarcity of the oil [1]. Algae have long had the attention of biofuel investigators as a new source of oil for biofuel production as it can produce more oil compared to other biofuel feed stocks in shorter periods of time and in smaller areas. Algae are photosynthetic organisms which utilize solar energy to grow and convert water and carbon dioxide into lipids and other metabolites. They also can be grown on waste water generated by the agricultural, food industry [2]. Some researchers also identified the possibility of using waste water generated from coal seam gas industry to grow algae [3]. Algae have been used as a source to produce a wide variety of natural products for pharmaceutical, biomedical, and nutraceutical industries. Carbohydrates, polyunsaturated fatty acids (PUFAs), vitamins, minerals, and dietary fibers are some of the commercial products derived from algae other than oils. Development and marketing of these byproducts are crucial for sustainable production of algae biodiesel and this strategy is widely known as 'algal bio refinery' [4,5].

Different methods have been demonstrated to produce biodiesel from microalgae. These processes involve drying of algal biomass and extracting oils with expeller press, solvent extraction, etc., [6,7] and some researchers have used supercritical CO_2 extraction of lipids to produce biofuels [8]. The extraction of oils is the most energy intensive step among the four steps; it consumes nearly 85% of production energy in the dry extraction method [9]. To eliminate the energy consumption involved in drying, wet processing methods have been explored to produce biofuels. Direct conversion of wet algal biomass to biodiesel was demonstrated using a supercritical ethanol transesterification method [10]. But due to limitations in scalability of this process, a large scale production is very difficult.

Subcritical water (SCW) extraction or hydrothermal liquefaction (HTL) is another way of isolating or producing necessary feedstock for biofuels. Water is identified as an environmentally benign, non-toxic medium, with selective extraction or reaction capabilities and is a readily available green solvent. The process of converting biomass in HTL will be performed at medium-temperatures (200–370 °C) and high pressure. The characteristics of bio-crude or crude extract produced during this process vary with process temperature and pressure. The solubility of organic matter begins to increase rapidly at about 200 °C, and this enhanced solubility for organic compounds is provided by a homogeneous single-phase medium for organic synthesis in subcritical water [11]. The reduction of the dielectric constant makes water a suitable solvent for small organic compounds, as its dielectric constant drops from 80 at 25 °C to 40 at 200 °C. Subcritical water extraction has been demonstrated for the extraction of mannitol from olive leaves [12], and essential oils from coriander seeds [13].

In hydrothermal liquefaction and at elevated temperatures, biochemical compounds present in the biomass undergo reactions like hydrolysis, repolymerization to form energy dense biocrude oil, bio-char, water soluble compounds and gaseous products. During this process, the oxygen present in the biomass will be removed by dehydration in the form of water, and by decarboxylation in the form of carbon dioxide [14,15]. Successful liquefaction of whole algae was demonstrated by Biller et al [16,17], Brown et al. [18] and Toor et al. [19] at temperatures of 300 °C or higher to produce an energy dense bio-crude oil. The major obstacle to refine the bio-crude oil in regular refineries is its higher nitrogen content, which requires special catalysts or processing strategies [19,20]. As discussed earlier, the commercialization of algal biofuels requires co-production of high value by-products from algae along with fuels. With whole algae conversion in HTL, the option of by-product has to be sacrificed in order to produce bio-crude oil.

To demonstrate algae bio-refinery and produce fuel, subcritical water extraction was chosen. This is the first study of this kind where lipids/oils can be extracted directly from wet algal biomass while preserving the valuable by-products. In this study along with conventional heating SCW extraction, microwave assisted SCW method for the extraction of lipids was performed. The heat transfer mechanism in conventional heating depends on the thermal conductivity of the solvent, and sample. The convective currents make it a slow process in conventional heating, where in microwave heating the volumetric heating makes the heat transfer process is fast and rapid [21]. Microwave-assisted extraction is a fairly new technology which has greater selectivity towards desired compounds and a faster and better recovering capacity than traditional methods [22]. In this novel process, the resistance offered by the solution to the passing electrophoretic migration of ions of electromagnetic field causes friction between molecules resulting in the generation of heat [23]. Along with external heat, the water inside the cell body evaporates and bursts cell walls making extraction of cellular contents much easier [24,25]. There have been successful demonstrations of the extraction of essential oils from the leaves of murraya koenigii [26], and plant materials [27].

In this study, extraction of lipids/oil was demonstrated through conventional heating subcritical water extraction (C-SCW) and microwave-assisted subcritical water extraction (MW-SCW). Both the processes are more selective towards lipids/oils. Preliminary observations provided the basis for central composite design, which was employed to study the effects of extraction temperature, extraction time, and biomass loading on crude extract yield. When optimum parameters were established for each method, extraction efficiency was determined and compared to a conventional solvent extraction method. Produced crude extracts were also analyzed by FT-ICR MS for qualitative compositional description. Lipid extracted algae (LEA) was analyzed for nutrient value and calorific value. The thermal behavior of algae, crude extract and pure algal oil samples was determined using a thermogravimetric analyzer (Perkin Elmer Pyris 1 TGA).

2. Experimental section

2.1. Materials and methods

Nannochloropsis salina algal biomass was received from Solix biofuels (Fort Collins, Colorado, USA) and NMSU Energy Research Laboratory. Moisture content was 62% and 63% for respective biomasses in above stated order and both were harvested by centrifugation. All solvents used in this study were analytical grade reagents.

PARR 4593 stainless steel bench top reactor accompanied by a 4843 controller unit manufactured by Parr Instrument Company (Moline, Illinois, USA) was used for conventional heating extraction experiments. The microwave-assisted subcritical water extraction experiments were performed in an Anton Paar multiwave 3000 microwave reactor (operating parameters: 0–60 bar, 25–220 °C, 0–1400 W, 10–60 mL/Teflon tube reactor with 16 tubes) enclosed with a specially designed rotor (Graz, Austria). Both reactors are equipped with pressure gauges. Imaging of thin sections of algae was carried out with a model H-7650 transmission electron microscope (Hitachi High-Technologies America, Pleasanton, CA). Thermo gravimetric analysis (TGA) of wet algal biomass was performed using Perkin Elmer Pyris 1 TGA (Perkin Elmer Inc., USA) instrument. A Hewlett Packard 5890 gas chromatograph with a 5972a mass selective detector equipped with a capillary column DB-23, 30 m × .25 mm diam. × .25 μm film was used for fatty acid methyl ester analysis. Compositional analysis of intact lipids was performed for lipid extracts by direct infusion into a hybrid linear ion trap FT-ICR mass spectrometer (LTQ FT, Thermo, San Jose, CA) equipped with an Advion Triversa NanoMate (Advion, Ithaca, NY).

2.2. Conventional lipid extraction

Conventional lipid extraction was performed by the Folch method [28]. For this procedure, dried algal samples (0.1 g) were extracted in triplicate for 30 min with 2 mL of chloroform/methanol (2:1 v/v) at 25 °C with continual vortexing. Extracts were centrifuged and the supernatant removed. Extraction was repeated and combined supernatants were evaporated in pre-weighed vials under a steam of nitrogen. All lipid extracts were stored under nitrogen at −20 °C for FT-ICR MS analysis.

2.3. GC/MS FAME analysis

FAME (fatty acid methyl esters) analysis was performed by direct methylation of 50 mg of dry tissue or crude extract which is weighed and placed in 12 mL glass tube. Next, 10 μL of glycerol tritridecanoate (13:0 FAME standard at 20 mg/mL in Hexane) as internal standard was added to each sample vial. Then 5 mL of 0.2 N KOH in MeOH was added and each sample was vortexed for 20 s. These samples were placed in hot water bath at 65 °C for ten minutes and vortexed for 30 s. These last two steps were repeated three times total. To stop the reaction, 1 mL of 1 M acetic acid was added to each sample and then vortexed each sample for 20 s. Two milliliters of Hexane with internal standard (methyl tricosanoate at 50 mg/L in Hexane) was added to each sample vial. Each sample was vortexed for 20 s and two phases are separated by centrifugation. The top hexane layer was taken for the GC–MS analysis. Helium was used as the carrier gas with a 2 μL injection volume. The temperature ramp started at 80 °C and ramped 20 °C/min to 220 °C and held for 6 min for a total run time of 13.3 min. The instrument was tuned with a standard spectra auto tune method, and a calibration curve was made from a Supelco 37 Comp. FAME mix 10 mg/mL in CH_2Cl_2. Total lipid was determined gravimetrically by the Folch method [28] and the lipid profile determined by FAME analysis as described above.

2.4. Direct infusion ESI FT-ICR MS and data processing

Samples were analyzed by direct infusion mass spectrometry performed with a hybrid linear ion trap FT-ICR MS (Fourier transform ion cyclotron resonance mass spectrometry) as recently described by Holguin [29]. Algal lipid extracts were prepared by dissolution in methanol:chloroform (1:2 v/v) for a normalized concentration of 10 mg/mL. These stock solutions were further diluted 200 fold into 1 mL of 2:1 methanol:chloroform for a final concentration of 0.05 mg/mL, which contained 5 μL of aqueous 1 M sodium acetate (positive ion mode) or 1 M ammonium hydroxide (negative ion mode) and 10 μg/mL of phosphatidylethanolamine, PE (17:0/17:0), as an internal standard. All solvents used were purchased from Sigma (St. Louis, MO) and were HPLC grade. Sample introduction was performed with an Advion Triversa NanoMate. Data was collected at a mass resolving power of $m/\Delta m_{50\%}$ = 400,000 (m/z 400) and 150 time domain transients were co-added prior to fast Fourier transformation and frequency to m/z conversion. Elemental compositions were assigned and searched against a list of lipids derived from the Lipid Maps database.

3. Experimental procedure

In all experiments constant volume of feed (80 mL for C-SCW and 60 mL for MW-SCW) was used with varying biomass loading. Samples were prepared according to the biomass loading by adding sufficient D.I water. Then the samples were fed into the reactor or reactor tubes, and the temperature maintained as per the experimental plan. The reactors were cooled down after completion of the experiment, and all the product mixture was transferred into a separation funnel and 15 mL of n-hexane was added. 5 mL of n-hexane was used to wash the agitator to free any crude extract adhered to it, and then was transferred to the separation funnel. The mixture was thoroughly stirred with disposable spatula and then left for 15 min to settling. Then water layer was collected for the material balance measurements and hexane layer containing crude extract and LEA was transferred to centrifuge tubes.

Crude extract and LEA biomass were separated in centrifuge tubes, which were operated at 3200 rpm for 5 min. The less dense hexane layer, which contains lipids along with other extracted compounds, was separated from LEA and transferred into a pre-weighed rotary evaporator flask. The rotary evaporator was operated at 70 °C under vacuum to remove the hexane from crude extract. The crude extract weight is calculated after subtracting the flask weight; it is then transferred into sample vials. The schematic of the subcritical water extraction process is shown in Fig. 1. To prevent oxidation of crude extract samples they were preserved at −5 °C until analyzed. After analysis, crude extract produced at optimum conditions was purified through an activated charcoal bed with hexane as eluent to get pure algal oil. The pictures of algal crude extract and pure algal oil are showed as Fig. A3 in supplementary information.

3.1. Experimental design

The purpose of the experimental design is to optimize the conditions for maximum lipid extraction from wet algal biomass. Response surface methodology is a statistical method used for optimizing the independent variables for maximum or minimum response. In this work, the independent variables are the following: extraction temperature (°C), biomass loading (%-wt. of biomass/wt. of water), and extraction time (min). After finishing the experiments, a suitable mathematical model was developed to predict the response based on the experimental factors. A 90% significance level was used for model development and analysis of variance. Complete analysis of variance (ANOVA) is done using Minitab v16.1.0 and the contour plots explaining the response surface were obtained using Matlab v7.12.0.635 (R2011a).

There was no extraction achieved at temperatures below 160 °C. The crude extract yield increased as the temperature was increased to 250 °C, but by-products start degrading to undesirable compounds in the extracted crude extract. Hence a temperature range between 160 °C and 250 °C was used to for optimizing the C-SCW extraction process. The C-SCW extraction experiments were conducted with *N. salina* algal biomass procured from Solix biofuels to obtain the optimum conditions for maximum extraction of lipids/oils. Freshly cultured *N. salina* algal biomass, harvested from the NMSU photo bioreactor facility was used for microwave-assisted extraction experiments and Folch extraction. Same biomass was used to perform experiments of C-SCW at optimum extraction conditions, results were compared with MW-SCW at optimized conditions and Folch extraction for extraction efficiency. Due to limitations in operating conditions of the microwave system, the microwave-assisted subcritical water experiments are conducted between the temperature range 160 °C and 220 °C. Preliminary studies indicated that maximum crude extract yield is achieved during an extraction time from 15 to 30 min. Circumscribed central composite design was used to design the experiments between the experimental factors such as extraction temperature, extraction time, and biomass loading at three levels; low (−1), central (0), and high (1). This type of central composite design uses points outside the design space (−1.68, 1.68 levels) which provide a good estimate over the entire design space. There are total of 20 experiments for C-SCW extraction and 16 experiments for MW-SCW extraction, which are completely randomized to eliminate any systematic errors and shown in *Tables* A1 and A2 of appendix.

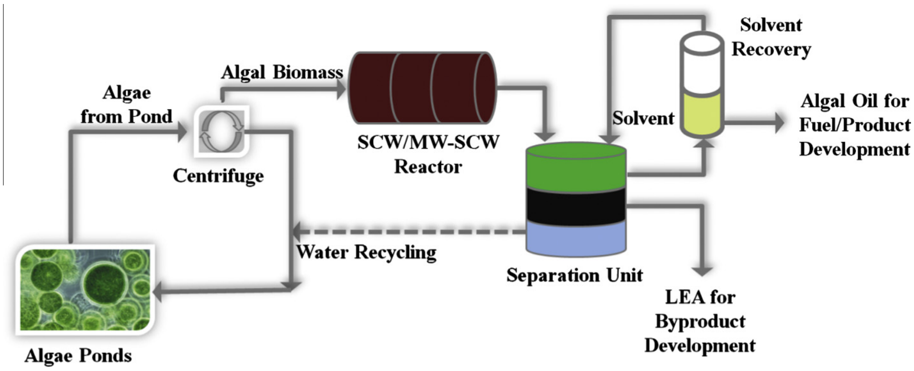

Fig. 1. Process protocol of subcritical water extraction from wet algal biomass.

3.2. Calculations

All the experiments were replicated five times at optimum conditions for consistency of the results and the average values of these replicates with standard deviation have been reported. The extraction of crude extract calculations are performed using the formulae given below:

$$\text{Crude extract yield} = \frac{\text{weight of crude extract}}{\text{dry weight of biomass}} * 100 \qquad (1)$$

$$\text{FAME (or) lipids yield in crude extract} = \frac{\text{weight of FAMEs}}{\text{weight of crude extract}} * 100 \qquad (2)$$

$$\text{FAME (or) lipids (\%) extracted} = \frac{\text{Avg. weight of FAMEs in crude extract}}{\text{dry weight of biomass}} * 100 \qquad (3)$$

$$\text{Extraction efficiency} = \frac{\text{Avg. Amount of FAMEs (or) neutral lipids extracted}}{\text{FAMEs content of the biomass (dry basis)}} * 100 \qquad (4)$$

4. Results and discussion

4.1. Subcritical water extraction of crude extract and lipids from wet algal biomass

Both the C-SCW and MW-SCW extraction methods have been carried out according to the design of experiments, and process parameters were optimized for maximum crude and lipid extraction. The optimized process parameters have been taken as standard process parameters for the final experiments and the extraction results have been compared to the conventional solvent extraction method i.e., Folch extraction.

A general linear model is used to model the crude extract yield as a function of extraction temperature, extraction time, and biomass loading for subcritical water extraction. In the model μ is the predicted response, x_1, x_2, and x_3 are experimental factor levels.

For C-SCW process, the process is explained by,

$$\mu = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij}x_i x_j + \sum_{i=1}^{3}\beta_{iii}x_i^3 \qquad (5)$$

The model fits the data very well with an R^2 value of 0.9357. The lack-of-fit of the model is insignificant with a p-value 0.38 and the overall model is significant with a p-value of 0. All the parameters in the model except the coefficient for biomass loading are significant at 90% significance level. The analysis of variance is presented in Table 1 and model coefficients in Table A3 in the supplementary information. According to the model, the maximum crude extract yield will be obtained at extraction temperature at 217 °C. The crude extract yield increases when the biomass loading decreases. Dewatering (dehydration) is also an energy prohibitive step if the biomass is recovered with or above 20% biomass loading, and it is preferable to use biomass loading between 7.5% and 20% [30] for wet extraction. For this reason, biomass loading of 7.5% is chosen as the optimum biomass loading. After considering all these factors, the optimum process conditions are fixed at 220 °C extraction temperatures, 7.5% biomass loading, and 25 min. extraction time and pressure observed was 24.5 bar at these conditions in optimized experiments.

For MW-CSW process, the process is explained by,

$$\mu = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 \qquad (6)$$

The model fits the data very well with an R^2 value of 0.8756. The overall model is significant with a p-value of 0.001. All the parameters in the model are significant at 90% significance level. The analysis of variance is presented in Table 2 and model coefficients in Table A4 in the supplementary information. After analyzing these results, 205 °C as extraction temperature, 25% biomass loading and 25 min. of extraction time are preferred as optimum process parameters. Pressure noted at optimum conditions is 21.5 bar. The RSM contour plots for both the processes are shown in Fig. 2.

4.1.1. Influence of process parameters in C-SCW experiments and response surface analysis

The contour plots of results shown in Fig. 2 demonstrate the influence of the parameters. Extraction temperature is the major influencing factor on crude extraction, which varies the polarity of the water [11,31]. Fig. 2a shows that extraction yield is increased

Table 1
Analysis of variance for crude extract yield using C-SCW extraction.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	6	1816.17	1816.17	302.69	31.521	0.000
Temp	1	468.79	325.2	325.20	33.864	0.000
Time	1	6.93	36.15	36.15	3.764	0.074
Biomass	1	29.46	16.16	16.16	1.683	0.217
Temp * temp	1	1060.68	266.97	266.97	27.801	0.000
Time * biomass	1	30.62	30.62	30.62	3.188	0.098
Temp * temp * temp	1	219.69	219.69	219.69	22.877	0.000
Error	13	124.84	124.84	9.60		
Lack-of-fit	8	85.65	85.65	10.71	1.366	0.381
Pure error	5	39.19	39.19	7.84		
Total	19	1941				

with the increase in the temperature until it crosses the optimum region border and this is attributed to the decreased polarity or increased solubility of water. The increased solubility of water at this temperature is caused by breaking of hydrogen bonds between water molecules which increases the miscibility between lipids and water [14]. This property of water also makes the separation very easy when the process temperatures are reduced to room temperature. Hydrolysis of TAGs is observed at 240 °C in the crude extract samples obtained in preliminary experiments and samples processed above 215 °C in the designed experiments. Hydrolysis is the predominant reaction in this region of temperature, and intention of this work is to extract lipids/oils in their original form while preserving the byproducts, hence lower temperatures are preferable for selective extraction of lipids.

The systematic optimization of temperature range is established for the specific extraction of lipids with this design, which is necessary for the extraction of respective class of compounds [32]. Dewatering is also an energy intensive step in algal biofuels production [30], therefore biomass loading has been examined as a parameter. Biomass loading is the second major influencing parameter in subcritical water extraction process. Fig. 2b shows the effect of biomass loading on the extraction of crude extract yield. The extraction of crude extract is increased with decreasing biomass loading and reached a maximum at 5% of biomass loading. The lesser biomass loading or higher solvent ratios, i.e., water ratios increases the liquid yields because of the denser solvent environment [31]. Extraction time is the last influential parameter on extraction of crude product. The yield of crude extract was increased from beginning of the extraction process and reached maximum around 30 min and the effect of extraction time on crude extract yield is shown in Fig. 2c. Even though 25 min. of extraction time is chosen as optimum time for extraction, as extended extraction times may cause a decrease in the bio-oil or bio-crude yield due to secondary and tertiary reactions like hydrolysis and re-polymerization, which are always crucial factors in hydrothermal liquefaction of biomass as they convert heavy compounds into liquids, gases or solid residues [31,33].

4.1.2. Influence of process parameters in MW-SCW experiments and response surface analysis

The effects of the process parameters extraction temperature, biomass loading and extraction time showed similar effects in MW-SCW as in SCW with small changes in those effects. The central composite design which used for the experiments and results are shown in Table A2. The extraction temperature is the main influencing parameter, as in conventional heating method. The crude extract yield is increased from 160 °C till the optimum region and is shown in Fig. 2d. Similar results were reported by Tsubaki et al. [34], where the extraction of phenolic compounds is increased with the increase in temperature. The decrease in dielectric constant or decreased polarity is caused by the breaking of hydrogen bonds between water molecules at higher temperatures which increases solubility of lipids in water [14].

The following influencing parameter after temperature is biomass loading. Differing with the conventional heating method in microwave heating method the yield of crude extract is increased with increasing biomass loading from lower amounts. Contour plot showed in Fig. 2e revealed that the optimum extraction is achieved at 25–29% biomass loading and 25% is chosen as the optimum value. The higher amount of solvent may decrease the extraction yields, because of the less stirring of the solvent by microwaves [23]. The higher solvent volumes decrease the probability of penetrating microwaves through the biomass may also be another reason for the decrease in extraction yields at lower biomass loading. Extraction time is the next influencing parameter on the crude extraction. Fig. 2f shows that the crude extract yields are increased with an increase in time through the optimum region, but further increase in extraction time decreased the crude extract yield. As discussed in conventional heating method the secondary and tertiary reactions seems to be the reason for the decrease in crude extract yield at prolonged extraction times. Even with 25 min. of extraction time the more amounts of water soluble compounds are extracted from the algae in MW-SCW extraction.

Table 2
Analysis of variance for crude extract yield using MW-SCW extraction method.

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	6	528.124	528.124	88.021	10.56	0.001
Linear	3	329.541	263.114	87.705	10.52	0.003
Temp	1	110.562	36.783	36.783	4.41	0.065
Time	1	150.668	154.811	154.811	18.57	0.002
Biomass	1	68.311	74.573	74.573	8.95	0.015
Square	3	198.582	198.582	66.194	7.94	0.007
Temp * temp	1	34.712	33.226	33.226	3.99	0.077
Time * time	1	113.559	112.049	112.049	13.44	0.005
Biomass * biomass	1	50.312	50.312	50.312	6.04	0.036
Residual error	9	75.018	75.018	8.335		
Total	15	603.142				

4.2. Extraction efficiency and energy consumption

This research was intended to achieve maximum extraction of lipids with less energy spending for extraction. The subcritical water extraction methods were compared to established Folch extraction method in terms of their extraction efficiencies based on the total FAME content of a direct transesterification of the algal biomass. All the optimized experimental results are an average of five replicates data of same conditions. Both the C-SCW and MW-SCW extractions displayed higher crude extract yields and lipids (as determined by FAME quantitation and gravimetric measurement) than the Folch extraction method. Among the three methods, the MW-SCW method extracted more crude extract and maximum lipids than the other methods. The conventional SCW extraction is able to extract nearly 70% of the lipids from the wet algal biomass. The conventional Folch extraction is able to extract only 33% of lipids present in the algae. The yields of the crude extracts and lipids extracted in the three methods are shown in Fig. 3.

The energy calculations at optimized conditions were performed with enthalpies of water as a saturated liquid under pressures [35]. For processing 1 kg of dry algae 13.34 kg wet paste is required (7.5% solids) in C-SCW extraction. Enthalpies of saturated liquids at 25 °C, and 220 °C are 0.104 MJ/kg, and 0.943 MJ/kg respectively. The Δh_f of the SCW process: $\Delta h_f = 0.943 - 0.104 = 0.839$ MJ/kg. Assuming the heat capacity of 1 kg dry algae is 50% of water, $\Delta H_{C\text{-}SCW} = 0.839\ (12.34\ \text{kg}) + 0.5\ (0.839)\ (1\ \text{kg}) = 10.77$ MJ/kg. For MW-SCW $\Delta H_{MW\text{-}SCW}$ is 9.89 MJ/kg. For processing 1 kg of dry biomass 4 kg of hexane should be used to separate crude extract and consider the latent heat of evaporation as 0.365 MJ/kg. The energy required to separate hexane is $4\ \text{kg} \times 0.365\ \text{MJ/kg} = 1.46$ MJ, which makes the total energy required for processing 1 kg of biomass $10.77 + 1.46 = 12.23$ MJ. The SCW extraction reduces the energy consumption for extraction calculated by Khoo et al., (152 MJ/kg) [9] by two times to produce 1 kg of biomass. With energy recovery (50%) from hot streams in SCW process and energy from LEA (60%), the total energy consumed can be further reduced by 8 folds.

C-SCW extraction appears promising for extraction of lipids within the existing industrial infrastructure, as it operates at moderate temperatures and pressure than HTL. By using little more hexane for separation might separate the remaining lipids from

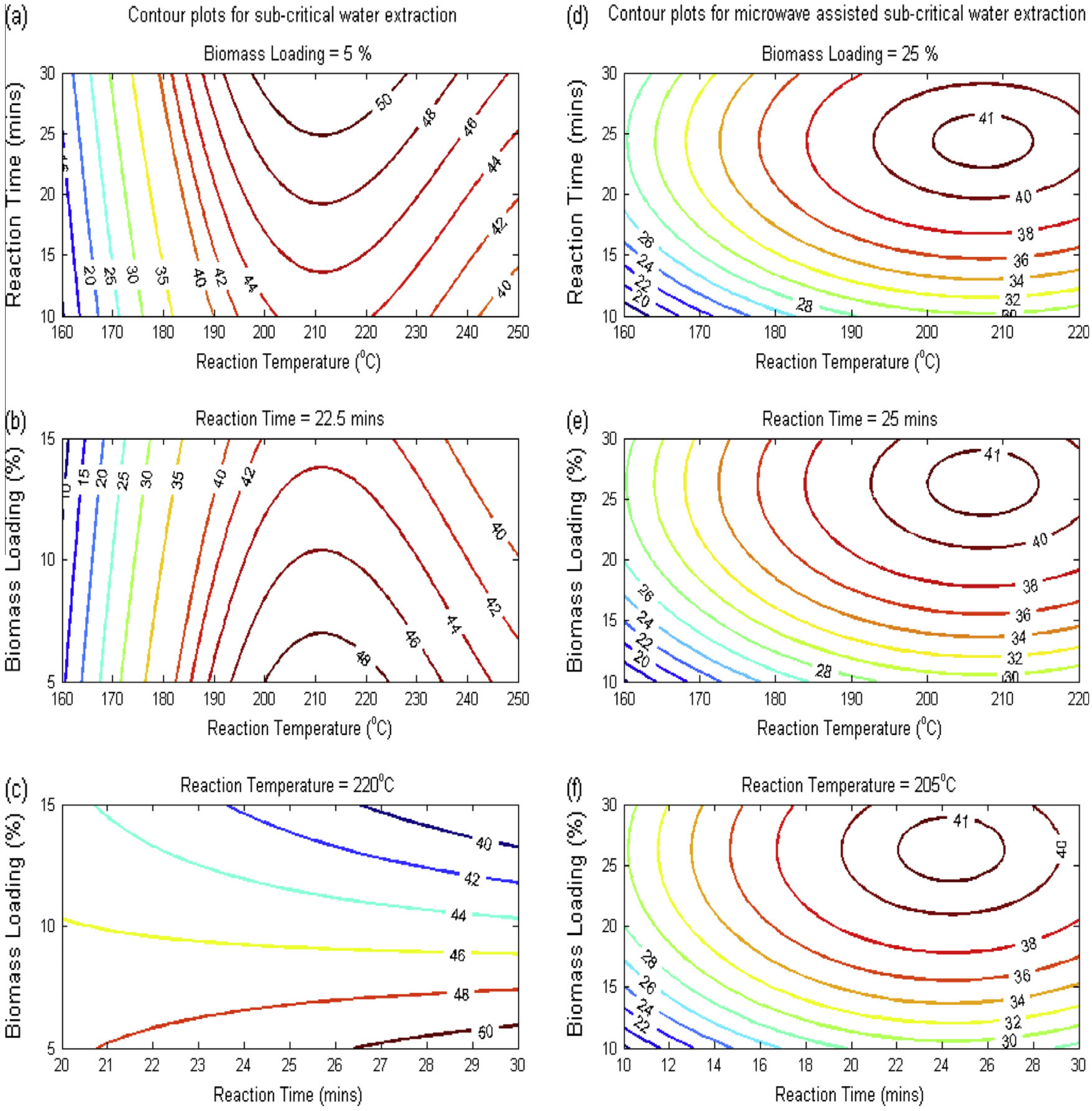

Fig. 2. Contour plots of experimental parameters on crude extract.

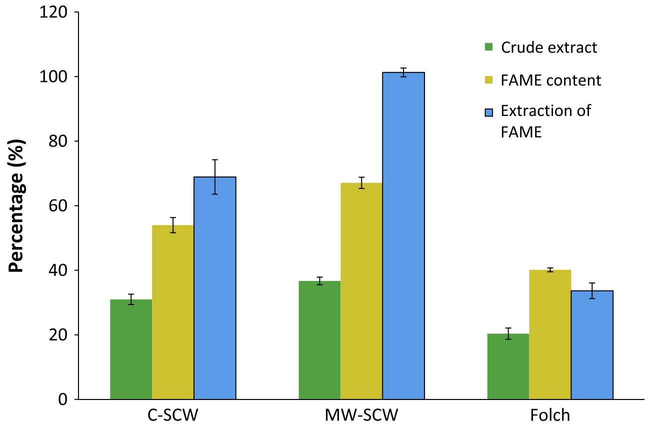

Fig. 3. Yields of crude extract, lipid content in crude extract and extraction efficiencies of respective methods.

LEA. Even though complete extraction of lipids was achieved in MW-SCW, at present there are no available industrial scale microwave technologies available to scale up this method. This method could be used as laboratory scale extraction method for analytical purposes as it reduces the sample preparation like drying, etc., The major obstacles for scale-up of microwave systems are low penetration depth of microwaves radiation at large scales, which creates thermal discontinuity throughout the reactor. By using of high power magnetrons this problem can be overcome, but needs oil or water cooling, which makes the instrument much bulkier and costlier [36].

Along with lipids, the lipid extracted algae (LEA) is also an important byproduct that needs to be quantified. The weights of LEA and water soluble compounds were carefully noted after every experimental run at optimized conditions. 27 ± 1.3% of LEA and 39 ± 0.9% of water soluble compounds in C-SCW and 12 ± 1.3% LEA and 51 ± 1.7% water soluble compounds in MW-SCW were observed. These compounds have commercial demand and extraction from algae needs to be studied thoroughly. In conventional heating method more LEA biomass is retained compared to the MW-SCW method. This could be a result implicated from the direct heating of the biomass through the water medium, in the later method the reaction temperature is achieved by microwave heating. In an opposite manner, more water soluble compounds are extracted in MW-SCW than conventional heating method. The preliminary nutrient analysis and calorific values of LEA also prove that MW-SCW has extracted most of the compounds both in crude extract and water phase. The preliminary analysis of water showed the presence of sugars (glucose, melezitose), sugar alcohols (mannitol, galactitol, and sorbitol), but needed more detailed analysis for quantification. Recently, Chakraborty et al., demonstrated the extraction of polysaccharides at a lower temperature as part of sequential HTL of *Chlorella sorokiniana* algal biomass [37].

4.3. Biomass and crude extract analysis

4.3.1. Biomass characterization

The freshly harvested *N. salina* from photo bioreactors has been analyzed for its fatty acid profile by direct FAME method described in the analytical methods section. The biomass has 24.3% (dry wt. basis) of FAMEs or neutral lipids. The major fatty acids detected are hexadecanoic acid, C16:0 (29.71%); hexadecenoic acid, C16:1n7 (41.91%); cis-9-Octadecanoic acid, C18:1n9c (9.9%); cis-5,8,11,14,17-Eicosapentenoic acid, C20:5n3 (6.78%) acid along with small fractions of other fatty acids.

The SEM-EDS analysis of LEA samples revealed that there is a substantial increase in carbon (79–86%) and decrease in oxygen (17–11%) content in biomass after extraction in conventional heating method, which is caused by the hydrothermal carbonization of the biomass. Hydrothermal carbonization of micro algae and prairie grass is demonstrated by [35] to produce char which has nearly equivalent energy to the bituminous coal. In MW-SCW method the exact opposite results have been found, where the LEA has less carbon and more oxygen than former method. The TEM analysis of biomass before and after extraction revealed the difference in both extraction methods. In feedstock biomass, the lipid globules are observed inside the biomass and marked with white dashed arrows in Fig. 4a. In conventional heating SCW method, some of the lipid particles are observed outside the cell walls and between the cells which shown in Fig. 4b, which shows the remaining lipids after extraction. This might be because of the strong adsorbing nature of LEA, which seems like charcoal. The lipid globules are marked with black dashed arrows and algal char is marked with white dashed arrows. But in case of MW-SCW method LEA there are only few organelles are present inside the algal cells showed in Fig. 4c., and empty space created inside the cells are marked with black arrows. This is the indication of the extraction capacity of both crude extract and water soluble compounds in MW-SCW method.

4.3.2. Crude extract characterization

Crude extract samples extracted at optimum conditions both in conventional SCW extraction and MW SCW experiments are analyzed with GC–MS for fatty acid profile and FT-ICR for lipid profile. The lipids extracted in Folch extraction are also analyzed and compared to the SCW methods. All extracts are thick green in color and slightly viscous in nature. The purified algal oil has a golden-yellow color with a sweet aroma. The purified algal oil has same fatty acid profile of the respective crude extract samples. The fatty acid profiles of crude extract samples obtained in each method at optimized conditions, along with Folch extraction were shown in Fig. A1 in appendix. The intact lipid distribution acquired by FT-ICR MS (Fig. 4d) show distinctly that the SCW water methods are relatively deficient in polar lipids such as 1,2-diacylglyceryl-3-*O*-4'-(*N,N,N*-trimethyl)-homoserine (DGTS), mono and digalactosyldiacylglyerols (MGDG, DGDG), phosphotidylcholines (PC), phosphotidylserine (PS) and phosphotidylglycerols (PG). The Folch extracts show greater diversity of lipid species while both SCW extraction methods indicate an enrichment of triacylglycerols. The conventional SCW method showed an increase of di- and monoacylglycerols. The increased ratio of DAG to TAG indicates partial hydrolysis of glycerol lipid acyl chains under these conditions. The analysis of the negative ion mass spectra supports the observation of hydrolysis by detection of free fatty acids (Fig. 4e). The MW-SCW extraction showed higher amounts of phosphotidylinositiol (PI) and vitamin E than either the conventional SCW or solvent based Folch extraction. Of interesting note is limited detection of C40 isoprenoids in the both the MW-SCW and SCW extracts. Along with the neutral lipids there are minor quantities of alkanes, nitrogen containing cyclic compounds and other compounds derived from algae metabolites are observed in the remaining portion of biocrude oil.

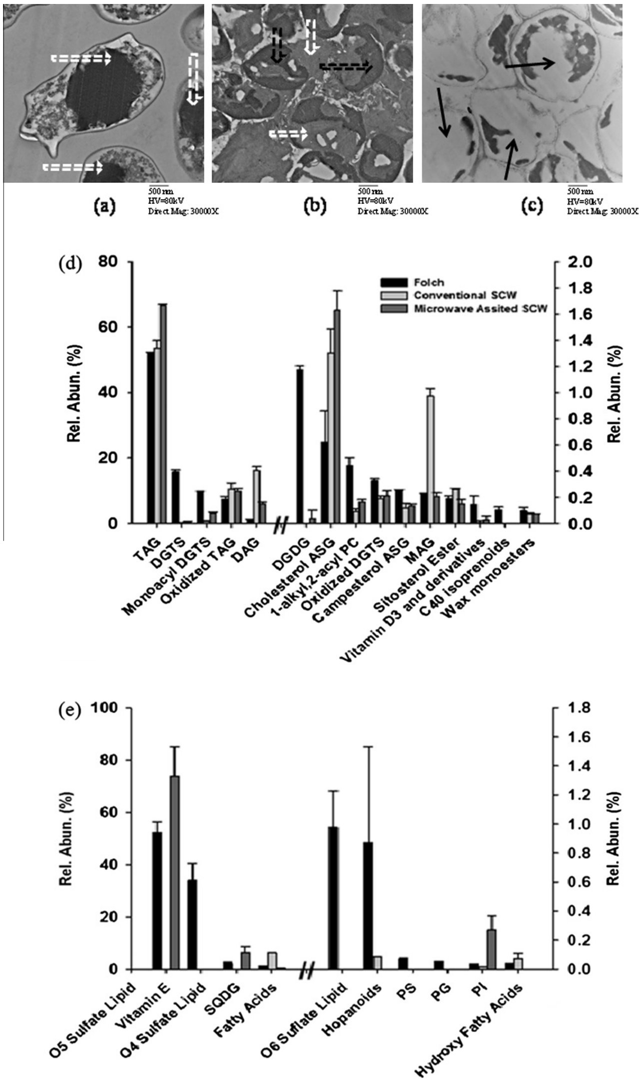

Fig. 4. TEM images of algal biomass (a) fresh algal biomass; (b) conventional SCW LEA; (c) MW-SCW LEA; FT-ICR analysis of lipid extracts; (e) positive ion ESI; (f) negative ion ESI.

4.3.3. Thermal behavior of algal biomass and crude extract

Thermo gravimetric analysis (TGA) of wet algal biomass, extract and purified algal oil is performed to analyze the thermal behavior of biomass, crude extract, and algal oil. The samples are heated from 25 °C to 950 °C at a constant heating rate of 10 °C/min in nitrogen atmosphere and at a constant purge rate of 20 mL/min at the pan. The linear plots of TGA for wet algal biomass, crude extract, and pure algal oil are shown in Fig. A2.

The biomass has undergone three phases of weight loss, one between 100 and 140 °C, the second at 250–300 °C and a third loss around 350–500 °C. The first shift represents weight loss caused by dehydration of the biomass sample. Nearly 60–65% weight is reduced in the first shift of physical change representing evaporation of water content in the sample. The second and third weight shifts are attributed to losses of organic compounds and decomposition of the algal biomass [38]. Apart from biomass, the crude extract missed the first shift of weight loss and does not have any incombustible material as it has only the extracted compounds. The last and third plot of pure algal oil is similar to regular vegetable oils and the only difference is a small weight loss between 250 °C and 300 °C. The thermal behavior of pure algal oil is close to the camelina oil [39] and matched with crude algal lipids [40]. As mentioned earlier, the polyunsaturated fatty acids present in the algal biomass are degrading in this range, and it can be observed in all three samples. The dotted circle shows this phenomenon in algal biomass, crude extract and in pure algal oil and can be attributed to degradation of polyunsaturated fatty acids in the samples.

4.4. Development of byproducts

As discussed earlier, the development of valuable byproducts is necessary for the sustainable production of algal biofuels. One valuable byproduct is eicosapentaenoic acid (EPA), which is an omega-3 fatty acid with medicinal applications like treatment of certain coronary heart disease, blood platelet aggregation, and abnormal cholesterol levels [41]. In our C-SCW process, the EPA is in the form of free fatty acids which can be easily separated before the conversion of algal oil into fuels. The LEA nearly has 24.7 MJ/kg HHV (high heating value) after extraction and 45.6% of crude protein in conventional heated SCW. This high HHV due to remaining lipids present in LEA and protein content. This lipid content should be separated to produce more oil which increases the overall extraction efficiency. The LEA produced from MW-SCW has 28.5% crude protein and 21.9 MJ/kg of HHV. The LEA produced in both methods has very high amounts of crude protein after extraction, which makes it a good animal feed source. Otherwise, the recoverable protein can be extracted for commercial human food applications as per the demand. The LEA can also be fired along with coal in power generation because of their higher calorific values. The gasification of the LEA could be used to make syngas, which can be further converted into valuable industrial chemical products [42]. Last but not the least is the residual water, which contains sugars (glucose, melezitose), sugar alcohols (mannitol, galactitol, and sorbitol), and nutrients (NH_3-N and PO_4^-) recovered from algal biomass. Recent literature also suggests that this water could be used to cultivate algal biomass with adequate dilutions [43]. The recovery of these dissolved organic compounds and nutrients provides potential byproducts in the bio refinery process.

5. Conclusions

The extraction of algal lipids was performed under subcritical water conditions from wet algal biomass. The results achieved in subcritical water (C-SCW and MW-SCW) extraction processes are far superior to those obtained with the conventional solvent extraction method. The subcritical waster extraction process is energy efficient because it eliminates the drying of wet algal biomass required in the conventional solvent extraction processes. Experimental results revealed that the lipid extraction efficiency of 70% in C-SCW and 100% in MW-SCW methods was achieved, while reducing extraction energy by 2–8 times. Extraction temperature (220 °C, 205 °C), biomass loading (7.5%, 25%) and extraction time (25 min. for both) are optimized for a maximum extraction of lipids in C-SCW and MW-SCW methods. The SCW extraction process provides a much-needed solution for the commercialization of algal biofuels at lower costs along with options to develop byproducts.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fuel.2014.04.081.

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