The effect of high-intensity ultrasound on cell disruption and lipid extraction from high-solids viscous slurries of *Nannochloropsis* sp. biomass

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Key Words: *Nannochloropsis* sp.; cell rupture; ultrasonication; speed of sound; lipid extraction; algae biofuel

Abstract:

The effect of ultrasonication on the cell rupture of marine microalgae *Nannochloropsis* sp. was studied as a function of the slurry solids concentration and treatment time. The concentrated viscous wet-biomass (~12 to 25% solids concentration) was subjected to ultrasonication (20 kHz) at 3.8 W/mL for up to 5 minutes. Compared to extraction without cell rupture, sonication led to a significant increase in lipid yield from ~11% to about 70% within 5 minutes of sonication. The extraction yield was found to decrease with increased solids concentration, with a large decrease between 20% to 25% solids. This is attributed to the increase in viscosity and decrease in speed of sound with increase in solids. The ultrasound attenuation coefficient increased 320-fold as the solids increased from 20 to 25%. Such a large attenuation of ultrasound places a limit of 20% solids to be used for cell rupture by ultrasound. The specific energy requirements per unit mass of extracted lipid were lowest at 20% solids. At lower concentrations energy was wasted heating water, at higher concentrations the lipid yields were reduced due to ultrasound attenuation.

1. Introduction

It is important to minimise the cost of producing and processing microalgae biomass to enable economically viable production of commodity products such as biofuels and protein feeds [4]. The use of novel processing techniques that consume less power than conventional techniques could be one of the strategies to reduce the overall processing cost. The processing of algal biomass to biofuels involves many energy-intensive steps such as harvesting, cell rupture, solvent extraction and the conversion of lipids to biofuels by transesterification or hydroprocessing. Energy efficient production of biofuels is possible only when the energy consumption of each of these processes is minimised. [3, 4, 6]

Oil extraction from conventional crops such as soybeans is performed on essentially dry biomass. However, algal biomass is harvested at low concentrations (e.g. 0.5 g/L) and can only be mechanically dewatered to about 25-30% solids [10]. Conventionally, lipids are extracted from dry biomass, however complete drying of the algae biomass prior to lipid extraction is not feasible as it is energy intensive and costly [10]. Therefore, extraction of lipids from concentrated slurries of biomass (~20% solids or more) is required [6]. As the lipids are stored inside the algae, the cells walls need to be ruptured for effective lipid extraction [11]. Various methods are available to rupture the cell walls, including high-pressure homogenisation (HPH),

bead beating (BB), microwave (MW), ultrasonics (US), hydrodynamic cavitation (HC), and heat/sulfuric acid treatment [12]. Of these techniques, only HPH has so far been demonstrated to be energy efficient and scalable [13]. HPH is able to effectively process concentrated slurries (~20% solids) of biomass [13, 14] and rupture the cells of various microalgae species [15]. Although HPH is energy efficient and scalable, there is the possibility of it being susceptible to clogging or damage by foreign debris in the biomass. [16] In HPH, the fluid is pumped at high pressure through a microvalve, which can be damaged or blocked by small, solid particles are present in the cell slurries, resulting in increased maintenance costs [16].

Ultrasonication is a possible alternative to HPH that is not susceptible to damage or clogging in the presence of particles. Like HPH, ultrasonication can be scaled up and used in a continuous mode. There are a number of large-scale flow through sonication systems that are available commercially and currently being developed to process volumes on the scale of a biorefinery [16]. Ultrasonic-induced microalgal cell rupture has been widely studied, but almost exclusively for dilute slurries as summarised in Table 1.

It has been shown that concentrated, rather than dilute slurries of biomass have to be processed in order for HPH to be energetically feasible [13]. It is likely that this is also the case for ultrasonic rupture, as in dilute slurries most of the ultrasonic energy is wasted heating up the solution rather than breaking the cells. However, the effectiveness of ultrasound has yet not been examined in detail on high-solids microalgal slurries. It appears that only one study, by Adam et al [17], has considered ultrasonication of high-solids slurries (5-30%) of microalgal biomass. However, the method used in the study resulted in extremely low lipid yields (<0.25%), the highest of which was obtained at the lowest slurry solids concentration (5% dry weight). Notwithstanding the minimal lipid yields obtained, the energy applied to the slurries was excessively high (3600 MJ/kg dry algae). As the efficiency of ultrasonication in rupturing cells depends on the acoustic attenuation through on the medium, optimizing the process requires a systematic characterization and understanding of the rheological and acoustic properties of microalgae slurries. However, there is a lack of studies that investigate the attenuation of ultrasound in high-solids microalgae slurries.

In this study, we investigated the effectiveness and the energy consumption of low-frequency high-intensity ultrasound in rupturing microalgal cells in concentrated slurries ranging from 12 to 25% solids. These results were compared to the established technology, HPH. For the first

time, we investigated the attenuation of ultrasound in concentrated algae slurries as a function of solids concentration by considering the high-shear viscosity during ultrasonication and the speed of sound through the sonicated-medium. We investigated a commercially relevant marine species, *Nannochloropsis* sp. that has been shown to be difficult to rupture [15]. This study will provide new insights into the practical utility of ultrasound in rupturing microalgal cells, based on its effectiveness and energy consumption in processing concentrated slurries.

Table 1. Summary of research on ultrasonication of slurries of microalgal cells.

Study	Species	Solids Concentration	Energy (MJ/kg dry mass)	Findings
Gerde et al.,[18]	<i>Schizochytrium limacinum</i> ; <i>Chlamydomonas reinhardtii</i>	1.4%	71	Beyond 100 J/ml of energy input there was not a significant improvement in cell rupture
Halim et al.,[19]	<i>Chlorococcum</i> sp.;	0.85%	132	The lipid yields were low (~14%)
Keris-Sen [20]	<i>Tetraselmis suecica</i>	0.025 %	1-70	The lipid extraction yield was increased by 50% for ultrasonically treated biomass
Lee et al. [21]	Mixed microalgae	0.5%	360	The lipid recovery was less than 10%.
Wang et al. [22]	<i>Chlorella vulgaris</i> ; <i>Botryococcus</i> sp.;	0.035%	-	The lipid recovery was less than 10%.
	<i>Scenedesmus</i> sp.			
Wang and Yuan [23]	<i>Scenedesmus dimorphus</i> ;	0.05-0.36%	10-75	It was concluded that HFFU (High Frequency Focussed Ultrasound) was more effective and energy efficient than LFNFU (Low Frequency Non-Focussed Ultrasound).
	<i>Nannochloropsis oculata</i>			The cell debris concentration and Nile red lipid fluorescence density increased showing the rupture of cells
Shen and Mao [24]	<i>Scenedesmus dimorphus</i> ;	-	-	With US, the lipid extraction efficiency was 20% whereas it was 5% for the control sample
	<i>Chlorella protothecoides</i>			
Prabhakaran and Ravidran [25]	<i>Chlorella</i> sp.; <i>Nostoc</i> sp.;	0.5%	-	Lipid yields using Bligh and Dyer method were twice as high for ultrasonically treated biomass
	<i>Tolypothrix</i> sp.			
Zheng et al.,[26]	<i>Chlorella vulgaris</i>	-	-	The lipid extraction efficiency using Bligh and Dyer method increased to about 15% for ultrasonic ruptured cells where as it was less than 5% for non-ruptured intact cells.
Natarajan et al.,[27]	<i>Tetraselmis suecica</i> ;	0.1-1.2%	-	
	<i>Nannochloropsis</i> sp.;			
	<i>Chlorella</i> sp.			
Grimi et al.,[28]	<i>Nannochloropsis</i> sp.	1%	-	

Greenly and Tester[29]	<i>C. reinhardtii</i> ; <i>Thalassiosira pseudonana</i> ; <i>Isochrysis galbana</i> ; <i>N. oculata</i> (CCMP525)	0.007-7.5%	-	Sonication was found to be more effective within first 30 s.
Adam et al. [17]	<i>Nannochloropsis oculata</i>	10-30%	600-3600	The extracted lipid amount was not quantified directly. The recovered FAMES (Fatty Acid Methyl Esters) were quantified by converting the lipids. The maximum amount of FAMES recovered was very low (about 0.25%).

2. Materials and methods

2.1 Microalgae biomass cultivation

A previously maintained [30] strain of *Nannochloropsis* sp. was used in the study. It was grown indoors in 15 L carboys at 20 °C with a light:dark cycle of 14:10 hours, using a modified f medium[14]. The bioreactors were aerated with aquarium air pumps (Stellar 380D, Aqua One, China) at a total flow rate of 190 L/h. The cultures were harvested and concentrated into pastes using a disc stack centrifuge (Separator OTC 2-02-137, GEA Westfalia, Italy) after 14 days of growth. Under these conditions, the nitrate levels in the cultures were typically depleted in 4-5 days. The solids concentration of the harvested paste was determined to be about 30% by oven drying at 60 °C.

2.2 Cell weakening by incubation

To weaken the cells prior to rupture (by high pressure homogenization or ultrasonication), the concentrated *Nannochloropsis* sp. cake was diluted to a thick slurry of *ca* 20-25% dry weight and then incubated at 40 °C for 24 hours, with mixing provided by an overhead stirrer motor (Stuart heat-stir CC162) at 70 rpm. The temperature was maintained using a water bath placed on a hot plate fitted with a thermostat (Heidolph R2R 2012 Control).

2.3 Solids concentration

Slurries with three different solids concentrations varying from 12 to 25% w/w were obtained by diluting a 30% slurry with 3% NaCl. The solids concentration was determined by oven drying at 60 °C until a consistent weight was obtained (48 hour drying).

2.4 *High intensity ultrasonication (US)*

To rupture *Nannochloropsis* sp. cells using ultrasound, 3 g of the incubated slurries were sonicated for 1, 3 or 5 min using a high-intensity ultrasonic transducer (Branson Sonifier operating at 20 kHz) with a micro-tip horn of 3 mm diameter. The bottom of the horn tip was positioned at the centre of the sample for equal distribution of power. During sonication, samples were placed in an ice-bath to ensure the samples were not overheated. The temperature of the samples after sonication was found to be 46.7, 54.4 and 58.7 °C respectively for 1, 3 and 5 minutes of sonication. Calorimetric studies were carried out by sonicating 100 mL of water for a specified amount of time and recording the temperature rise. Based on this, the power intensity used in our study was 3.8 W/mL. At least 3 replicates were performed at each condition.

2.5 *High-pressure homogenisation (HPH)*

As a comparison to ultrasound, a bench-top high-pressure homogeniser (GEA Panda 2K NS1001L with Re + valve; GEA Niro Soavi, Parma, Italy) was used to rupture the cells as previously described [15],[31]. Briefly, 100 g of the incubated slurries were passed once through the HPH at 1200 bar.

2.6 *Non-polar lipid extraction using hexane*

The effectiveness of cell rupture by ultrasound or HPH was assessed via the extraction level of non-polar lipids from the slurries using hexane [11]. This is based on the fact that nonpolar/hydrophobic solvents such as hexane are unable to diffuse across the cell wall effectively, and therefore the algal nonpolar lipids can only be extracted after the cell wall has been disrupted [11]. For this, a known amount of the ruptured-cell slurry (~1 g) was transferred to a glass vial with a liquid-tight lid and 5 mL of n-hexane (95%) was added. The hexane-biomass samples were mixed by rotating the vials for 72 hours at 8 rpm at room temperature. This was followed by 10 minutes of centrifugation at 1367 g (Beckman Coulter X-30R centrifuge) to separate the lipid-containing hexane from the dilipidated biomass. After centrifugation, there was a clear top layer consisting of a hexane-lipid mixture, a middle layer of emulsified biomass, and bottom aqueous layer. The top hexane-lipid layer was gently transferred to a glass vial of known weight. The collected hexane-lipid mixtures were dried under a stream of nitrogen at 60 °C using a Techne Sample Concentrator (model DB-3D Dri-Block, Bibby Scientific Limited, Stone, UK) to remove the solvent. Gravimetric analysis was used to determine the total quantity of the extracted non-polar lipids.

2.7 Bligh and Dyer method for total lipids extraction

To determine the total lipid content of the biomass a chloroform-methanol extraction was performed using a modified protocol from Bligh and Dyer[32]. For this, 1.2 mL of the *Nannochloropsis* sp. slurries (9-10% solids concentration) were added to 10 mL reaction tubes with screw-top lids, along with 3 mL methanol and 1.5 mL chloroform. The tubes were vortexed and then mixed overnight at room temperature. The following day, another 1.5 mL chloroform was added along with 1.5 mL water, the tube vortexed and then centrifuged at 1331 g for 10 min using a Beckman Coulter Allegra X-30R centrifuge. The aqueous methanol phase was removed from the top and the chloroform layer removed from the bottom. The chloroform layer was saved and the aqueous methanol layer discarded. The remaining biomass was resuspended to 1.2 mL with water, then 3 mL methanol and 1.5 mL chloroform were added. The tube was again vortexed and mixed for at least 2 h, following which the process was repeated until the biomass was colourless (generally at least four cycles). The extracts were dried under nitrogen flow at 60 °C using a Techne Sample Concentrator (model DB-3D Dri-Block, Bibby Scientific Limited, Stone, UK), weighed, and then resuspended in chloroform and aliquoted into appropriate vials for SPE (Solid Phase Extraction), GC, and HPLC analysis.

2.8 HPLC

To assess whether there was any lipid degradation due to sonication, lipids extracted by chloroform-methanol were analysed by HPLC-ELSD using a method developed by Graeve and Janssen [33] before and after being sonicated in 3 mL of hexane for 1, 3 and 5 mins (3.8 W/mL, 3 mm horn, 20 kHz). The lipid samples were dissolved in hexane, filtered through a 0.22 µm syringe filter, and analysed using an Agilent HPLC (1260 Infinity High Performance Micro Auto sampler) equipped with an ELSD (Evaporative Light Scattering Detector). An injection volume of 10 µL was used for the analysis. A Chromolith® Performance Si 100-4.6 HPLC column was used for the separation. A combination of three solvent mixtures were used as mobile phases. Mobile phase A consisted of isooctane and ethyl acetate (99.8:0.2, v/v), mobile phase B was a mixture of acetone and ethyl acetate (2:1, v/v) containing acetic acid (0.02% (v/v)), and mobile phase C consisted of 2-propanol and water (85:15, v/v) (with acetic acid and ethanolamine, each 0.05% (v/v)). A slight modification to the gradient program was made to achieve a similar separation to that previously described [33].

2.9 Solid Phase Extraction (SPE)

To determine the proportion of neutral lipids (NL), glycolipids (GL) and phospholipids (PL) present in the total lipid extract a solid phase extraction (SPE) was carried out as previously described [34], with the modification that nitrogen drying was performed at 50 °C.

2.10 Transesterification and GC-FID

To determine the fatty acids profile in the extracted lipids, the crude lipids were chemically transesterified and then analysed by gas chromatography with flame ionization detector (GC-FID). A known mass of triacylglyceride (C17:0 TAG standard, >99% glyceryl triheptadecanoate, Sigma, USA) was added to the dried lipid samples as an internal standard. 1 mL of chloroform:methanol (1:2, v/v) was then added to redissolve the lipids. Transesterification was performed by adding 0.15 mL of 10 wt% H₂SO₄ in methanol to the lipid solution and mixed (LabQuake, Barnstead International, Iowa, USA) for 3 hours at 55 °C. Subsequently, 0.5 mL of 25 wt% KCH₃O in methanol was added to the lipid solution and mixed for another 2 hours at 55 °C. Using a glass syringe and metal needle, the reaction mixtures were filtered through a 0.45 µm nylon filter (N66) into glass GC vials. The transesterified lipids were then analysed on a GC-FID (GC 7890B, Agilent Technologies, USA) with a DB-23 column (Agilent Technologies, USA). During the sample analysis, the GC oven was set to hold at 50 °C for 1 minute, then rise in temperature at a rate of 20 °C/min up to 180°C and then finally ramp to 210 °C at 2 °C/min.

2.11 High-shear viscosity and speed of sound measurements

The shear rate dependent viscosity was measured using a rheometer (MCR 702 Twin Drive from Anton-Paar) for 12%, 20% and 25% solids *Nannochloropsis* sp. paste. A vane-in-cup geometry (ST12.5 & C-CC17, Anton-Paar) was used for the viscosity measurement, with shear rates applied from 0.01 s⁻¹ to 200 s⁻¹. The speed of sound was measured by sending a single pulse of sound (10 µs pulse width generated by a wave form generator (Keysight, Model 33120A)) using an ultrasonic transducer through the algae slurry of known height (6.7 cm) and measuring the time taken by the pulse to reach the top of the algae slurry. The output signal at the top of the algae slurry is measured by a calibrated accelerometer (PCB Piezotronics, Model No: 353B17) driven by Signal Conditioner (PCB Piezotronics, Model No: 482) placed at the top of algae slurry covered with a tightly wrapped parafilm to prevent the contact of accelerometer with the algae slurry. The details along with the picture of the experimental setup is provided in Supplementary Information. The input pulse from the waveform generator and

output signal from the accelerometer were recorded using a digital storage oscilloscope (Keysight, Model No. DSO1052B). The time difference between these two signals can be used to measure the speed of sound. The average of 128 single pulses was used as a single experimental recording and it was repeated three times. The details of the recording for 3 experimental runs are shown in SI that show a good reproducibility. We first validated our experimental technique by measuring the speed of sound in water which was around 1492 ± 48 m/s compared to the literature value of 1498 m/s.

2.12 He-Ne Ion Beam Microscope imaging of ruptured cells

High resolution micrographs of intact and ruptured microalgae cells were obtained using a Helium Neon multi ion beam microscope (Zeiss, Orion NanoFab, Germany). To prepare the samples, diced Silicon wafers (13 mm by 13 mm squares) were coated with 0.1% (v/v) polyethyleneimine (PEI) by placing a drop of PEI on the wafer and drying by heating on a hot plate. The wafers were cooled back to room temperature and the cell suspensions (200 μ L) incubated on the PEI-coated silicon wafers for 1 h. After incubation, the excess cell suspension was drained, and the wafers with attached cells were immersed in 2.5% glutaraldehyde in PBS for 1 h to cross link the PEI film. Then the silicon wafers were rinsed three times in fresh PBS for 10 min each. The silicon wafers were then slowly dehydrated in increasing concentrations of ethanol consisting of 10, 30, 50, 70, 90 and 100% ethanol in water (10 min for each step). The silicon wafers were then stored in 100% ethanol. The wafers were dried in a critical point dryer (Balzers CPD030 Liechtenstein, Germany). The cells on the silicon wafers were imaged with the Helium-Neon Ion Beam Microscope with a spot size ranging between 4 to 500 μ m.

3 Results and discussion

3.1 The effect of ultrasonication on cell rupture

To investigate the cell rupture, a high resolution He-Ne multi ion beam microscope was used to image the before and after being subjected to ultrasonication (Figure 1). The images of the cells in the untreated suspension show that most of the cells are intact and are not ruptured (Figure 1a). The images of ultrasonic treated cells show an increase in cell rupture as the ultrasonication time increased from 3 to 5 minutes. The ruptured cell debris was aggregated, and there were some intact cells remaining after 5 minutes ultrasonication indicating that complete cell rupture had not been achieved.

Figure 1. Helium-Neon Ion microscope images of cells prior to ultrasonication (a), and after 3 min (b) and 5 min (c) of ultrasonication at 20 kHz and 3.8 W/mL at 20% solids concentration.

The ruptured cells as shown in figure 1 are likely the result of high shear forces and acoustic cavitation generated during the ultrasonication of the microalgae slurry. When ultrasound waves pass through a liquid medium, ‘acoustic cavitation’ occurs where pre-existing gas nuclei in the liquid medium are driven to oscillate due to fluctuating pressure field of acoustic waves [23, 35-37]. The cavitation bubbles grow due to rectified diffusion and collapse violently after reaching a critical size releasing large amounts of energy within the collapsing bubble. This results in the propagation of shockwaves in the medium generating high shear forces in the medium. Such extreme shear conditions could result in the breakup of the microalgae cell walls. The cavitation may occur within the individual cell resulting in the rupture of the cell when the bubbles collapse [38].

Ultrasonication may rupture cell walls through several mechanisms [38]. The first is through the cell wall membrane expansion and compression due to compression and rarefaction of ultrasound field. The cell membrane rigidity plays a greater role in this kind of rupture [39]. If the cell membrane is highly elastic, it can expand and shrink significantly without breaking. However, if the cell membrane is rigid it may break during the expansion. The second mechanism for cell rupture is through shear forces generated around the cell membrane due to acoustic cavitation outside the cell walls [40, 41]. One main possibility is the microjet breaking the cell wall when bubbles collapse on the cell wall. The third mechanism through is intramembrane cavitation where space between lipid monolayers inflates and deflates due to ultrasound [42]. The extent to which each of these mechanisms contributes to cell rupture is difficult to be predicted.

3.2 The effect of ultrasonic cell rupture on subsequent lipid extraction yield with hexane

The extent of cell rupture by ultrasonic treatment was evaluated in terms of the nonpolar lipid extracted using hexane. To investigate the effect of solids concentration on the effectiveness of ultrasonic pretreatment, experiments were performed at three different solids concentrations (12%, 20% and 25%). All the cell rupture experiments with sonication were performed at 3.8 W/mL (Figure 2). Unsurprisingly, the overall trend was for the amount of lipids extracted to increase with longer ultrasonic pretreatment times. The highest yield of 73.2% $\pm$ 8.4% was obtained after 5 min sonication on the least concentrated slurry (12% solids). For comparison, the extraction yield obtained from cells ruptured by HPH using a single pass at 1200 bar pressure was around 68% from a 20% solids *Nannochloropsis* sp. slurry.

Figure 2. Comparison of extraction yields achieved after pretreatment by ultrasound (US) at a power density of 3.8 W/mL for 1, 3 and 5 minutes. The extraction was done at room temperature for 72 hrs of tumbling. The comparison of extraction for three different solids concentration (12, 19.8 and 25%) and an untreated control are shown along with a slurry (18.7% solids) passed once through a high-pressure homogeniser (HPH) at 1200 bar. Error bars represent the standard deviation of triplicate experiments (n = 3).

For the control (no pretreatment) and the samples sonicated for 1 min, the extraction yields increased slightly as the solids concentration increased from 12 to 20% and then decreased with a further increase to 25% solids (Figure 2). For samples sonicated for 3 or 5 min, the extraction yields were similar at 12% and 20% solids and then decreased considerably at 25% solids. Such a decrease in efficiency can be attributed an increased attenuation of ultrasound waves when passing through a highly viscosity medium, which could result in less efficient cell rupture. Ultrasonic attenuation[43] is generally described through absorption (α) of sound waves by viscous liquids according to:

$$\alpha = \frac{2\mu(2\pi f)^2}{3\rho c^3} \quad (1)$$

Where α is the absorption coefficient, μ is the viscosity and ρ is the density of the medium, f is the frequency of ultrasound and c is the speed of sound in the medium. For a given frequency of ultrasound, the absorption coefficient increases linearly with an increase in viscosity. The

increase in density as a function of solids concentration is minimal compared to the increase in viscosity and decrease in speed of sound. As observed from figure 2, at longer sonication times, there was a decrease in extraction yield with an increase in solids concentration. High-solids slurries (19% solids) of *Nannochloropsis* sp. are shear thinning [44], so the shear forces generated by ultrasound will change the effective viscosity and hence the attenuation. The interactions between solids concentration, slurry rheology, ultrasonic attenuation and cell rupture were evaluated by measuring the slurry viscosity as a function of shear rate (Figure 3 (a)) and the speed of sound through algae slurry (Table 2). As observed from Figure 3(a), at all solids concentration, the slurries are shear thinning. Shear rate independent viscosity was observed around 200 s^{-1} . As the slurry is exposed to high shear forces during the ultrasonication, the high-shear viscosity at 200 s^{-1} (at which point it had reached a constant shear-independent viscosity) was used for estimating the ultrasound attenuation using equation 1. The high-shear viscosity of the 12% solids slurry was about 10 times that of water, while the high-shear viscosities of 20% and 25% solids algae slurry were about 85 and 15,000 times that of water respectively. The measured speed of sound through algae slurry decreased with increased solids concentration, as expected. The ultrasound attenuation coefficient (Table 2) increased 12-fold when the solids concentration was increased from 12% to 20% and it increased a further 320-fold from 20% solids to 25% solids. The significant increase in the attenuation coefficient is most likely responsible for the decrease in cell rupture as the solids concentration increases from 20 to 25% solids (Figure 2). From these observations, the maximum limit of solids concentration above which ultrasonication is no more effective for cell rupture is 20% due to significant ultrasound attenuation. To understand how sonication affects the viscosity of the paste, the shear viscosity behaviour of sonicated paste was measured at 20% solids (Figure 3(b)). As expected, the viscosity of the slurry increased with sonication due to the release of protein and other intracellular components. During sonication, as the cell rupture increases, the release of intracellular materials would continue to increase the viscosity and the ultrasound attenuation as a function of time. Quantifying this increase in attenuation during sonication is interesting but beyond the scope of the current study.

Figure 3. Shear viscosity of slurries of intact *Nannochloropsis* sp. cells as a function of solids concentration (a) and sonication ruptured *Nannochloropsis* sp. cells at 20% solids concentration (3 minutes sonication at 3.8 W/mL). The data shown in (b) were obtained using a different batch of *Nannochloropsis* sp. paste to the 20% solids data shown in (a). Triplicate measurements ($n = 3$) are shown for each data set.

Table 2. High-shear viscosity, speed of sound and ultrasound attenuation coefficient for slurries of intact *Nannochloropsis* sp. cells as a function of solids concentration. Error bars represent the standard deviation of triplicate experiments (n = 3).

Shear Rate (1/s)	Solids Concentration (% w/w)	Density (g/ml)	Viscosity μ (mPa.S)	Speed of Sound c (m/s)	Ultrasound Attenuation Coefficient $\alpha \cdot 10^4$
200	12%	1.061	9.6	1438 $\pm$ 19	0.32
200	20%	1.081	85.3	1274 $\pm$ 40	4.02
200	25%	1.100*	15010	1039 $\pm$ 38	1279.30

*extrapolated density

3.3 Analysis of lipids extracted after ultrasonic and high-pressure homogenisation pre-treatments

To investigate whether the method of pre-treatment (i.e. ultrasonication or high-pressure homogenisation) affected the composition of the lipids that were subsequently extracted by hexane, the FAME (Fatty Acid Methyl Ester) profiles were determined using GC-FID (Figure 4a) and the overall lipid class composition determined by HPLC (Figure 4b). The percent of total extracted lipids that could be converted to FAME was higher for the hexane extracts following HPH and sonication than for the total lipid extracts using the Bligh and Dyer method, reflecting a higher proportion of polar lipids, non-saponifiable lipids and pigments in the polar the Bligh and Dyer extracts. The proportion of saponifiable lipids were similar in the hexane extracts following HPH and sonication. The fatty acid profiles of the hexane-extracted lipids from the sonicated and HPH-treated biomass were comparable to each other (Figure 4b). In comparison to the Bligh and Dyer extracts, the lipids extracted following HPH/sonication were richer in C16:0 (palmitic acid) and C16:1 (palmitoleic acid), and contained a lower proportion of C20:5 (eicosapentaenoic acid). The FAME profile of the lipids extracted after HPH/sonication are consistent with the preferential extraction by hexane of TAG over polar lipids [14].

Figure 3. Comparison of the lipids extracted by the Bligh and Dyer method (B&D), to the lipids extracted using hexane after sonication (5 minutes) and high-pressure homogenisation (HPH) (1200 bar): (a) percent of extracted lipids convertible to fatty acid methyl esters (FAME); (b) corresponding FAME profiles of transesterified lipids. Error bars represent the standard deviation of duplicate experiments ($n = 2$).

To test the effect of ultrasonication on the lipids, samples of lipids pre-extracted by the Bligh and Dyer method and dissolved in hexane were sonicated for 1, 3 and 5 minutes at a power density of 3.8 W/mL. The lipid profiles were analysed using HPLC and seen to be unchanged. (Figure 4), indicating that ultrasonication did not hydrolyse the lipids.

Figure 4. Comparison of lipids extracted by Bligh and Dyer, dissolved in hexane and sonicated at 3.8 W/mL for 1, 3 and 5 minutes. Lipids were separated into lipid classes including sterol esters (SE), triacylglycerides (TAG), sterols, and free fatty acids (FFA) by high performance liquid chromatography. The lipid profiles of the non-sonicated and sonicated samples are identical. The inset is of an enlarged region that more clearly shows that the lipid profiles overlap. The results are representative of duplicate experiments that showed minimal variation.

3.4 Specific energy consumption

The energy consumed in rupturing microalgae cells and extracting the lipids can contribute significantly to the cost of biofuel production. Hence it is critical to compare the energy consumption of ultrasonication to more established technologies such as HPH. The energy consumption per unit of extracted lipid (based on the data presented in Figure 2) is presented for ultrasonication and HPH as a function of solids concentration (Figure 5). This analysis shows that the energy consumption of US treatment is considerably higher than HPH. However, when compared to energy consumption values for existing ultrasonic rupture of algae in the literature (Table 1), the energy consumption in our study is lower due to high solids concentrations that were used. Another way to calculate the specific energy consumption is the energy input per unit dry weight of the viscous biomass, which has units MJ/kg dry of cells. This is a monotonic decreasing function of the solids concentration; the higher the solids concentration the less is the energy needed. As discussed in the introduction section, in the case of US cell rupture of dilute cultures, most of the energy is used in heating up the water rather than for rupturing cells. Hence, the specific energy consumption (MJ/kg dry of cells) is very high. From an economic stand point, specific energy consumption in MJ/kg-lipid extracted is more appropriate because it is calculated relative to the amount of lipid extracted. However,

the relationship between the specific energy consumption (MJ/kg-lipid extracted) of ultrasonication and the solids concentration of algal slurry is not straightforward (Figure 5). It has been previously demonstrated that the effectiveness of HPH at rupturing algae cells is not changed as a function of solids concentration (in the tested range 0.25-25% solids)[13]. As the energy consumption of HPH is constant across this range of solids concentration, the energy per unit biomass (or lipid) is therefore reduced as a function of increasing solids concentration [13]. This was not shown here (Figure 5) but can be extrapolated based on this previous study. For ultrasound, while the energy input is similarly constant regardless of solids concentration, we have shown here that the effectiveness of the ultrasonic treatment does depend on solids concentration (Figure 2). Increasing the solids concentration from 12 to 20% resulted in similar lipid yields (Figure 2), which meant that the specific energy consumption was decreased due to intensification (Figure 5). However, a further increase to 25% solids, resulted in a decrease in lipid yield (Figure 2) that outweighed the intensification, resulting in a reduced energy efficiency (Figure 5).

Figure 5. Comparison of the specific energy consumption of ultrasonication (US) and high-pressure homogenisation (HPH) per unit lipid extracted. The data assume a biomass triacylglyceride (TAG) content of 20% and extraction recover yield based on the data presented in Figure 2.

Although the specific energy consumption of US is lowest at the shortest treatment time (1 min) (Figure 5), this is unlikely to be favourable due to the low lipid yields that were obtained (<40%) (Figure 2). Lipid yields in the 60-70% range were obtained using HPH and for

ultrasonic pre-treatments of 12% and 20% biomass for 3 and 5 min. Of these high lipids yielding conditions, the lowest specific energy for ultrasound was obtained at 3 min from 20% solids biomass. Here the specific energy consumption was 29.5 MJ/kg-lipid, compared to 4.4 MJ/kg-lipid for HPH. On this basis, it is unlikely that ultrasonication will be competitive with HPH for the pre-treatment of *Nannochloropsis* sp. unless it is found that clogging and maintenance issues for HPH are unresolvable.

As an additional comparison, the specific energy consumption of ultrasound (on 19.8% solids biomass for 5 min) and for HPH (on 18.7% biomass, 1 pass at 1200 bar) on the basis of dry biomass are 5.7 MJ/kg-dry biomass and 0.6 MJ/kg-dry biomass respectively. This translates to 1.6 kWh/kg-dry biomass and 0.17 kWh/kg-dry biomass, for ultrasound and HP respectively. On this basis, only HPH is below the NABTR (National Algal Biofuels Technology Roadmap, USA) [5] [NABTR Website] target of 0.633 kWh/ kg of dry algae for harvesting and extraction technologies.

4 Conclusions

High-intensity ultrasonication (20 kHz, 3.8 W/mL) was shown to effectively rupture the marine microalgae *Nannochloropsis* sp. in concentrated and viscous slurries, thus facilitating lipid recovery. Increasing the length of ultrasonic pre-treatment from 1 to 3 min greatly increased the cell rupture, while extending to 5 min had less effect. The effectiveness of the ultrasonic pre-treatment in rupturing cells was similar for slurries at 12% and 20% solids, but was significantly reduced at 25%, due to increase in ultrasonic attenuation in the highly viscous slurry. The measured high-shear viscosities increased with increase in solids whereas the speed of sound decreased. A large attenuation of ultrasound observed at 25% solids concentration places a limit of 20% solids on the cell rupture by ultrasound. The specific energy consumption of ultrasonic cell rupture was calculated and compared with HPH and other literature reported values. The condition with the lowest specific energy consumption that could achieve a high lipid yield (>55%) was ultrasonication of 20% solids biomass for 3 min. The specific energy consumption at this condition was 29.5 MJ/kg-lipid, about 6.5 times higher than HPH (4.4 MJ/kg-lipid). Nonetheless, this value was lower than the other reported values for ultrasonic pre-treatment due to the use of high solids concentration.

5 Acknowledgements

This work was performed in part at the Materials Characterization and Fabrication Platform (MCFP) and PFPC (Particulate Fluids Processing Centre) at the University of Melbourne. We thank the Australian Research Council (ARC) for providing funding through Discovery Projects Grant Scheme (DP170103791) and the University of Melbourne for providing infrastructure support. The authors would like to thank Dr. David R. A. Hill for his contribution in culturing and harvesting the microalgae. We thank Joshua Chung a Summer Research Exchange Program (SREP) student for his help with some of the experimental work.

Conflict of Interest Statement

No conflicts, informed consent, human or animal rights applicable.

Declaration of authors' contribution

All authors were involved in the conception and design of the study, analysis and interpretation of the data, and drafting of the article. All authors have given approval to the final version of the manuscript. G. Martin takes responsibility for the integrity of the entire work and can be contacted as gjmartin@unimelb.edu.au.

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Title:

The effect of high-intensity ultrasound on cell disruption and lipid extraction from high-solids viscous slurries of *Nannochloropsis* sp biomass

Date:

2018-11-01

Citation:

Yao, S., Mettu, S., Law, S. Q. K., Ashokkumar, M. & Martin, G. J. O. (2018). The effect of high-intensity ultrasound on cell disruption and lipid extraction from high-solids viscous slurries of *Nannochloropsis* sp biomass. *ALGAL RESEARCH-BIOMASS BIOFUELS AND BIOPRODUCTS*, 35, pp.341-348. <https://doi.org/10.1016/j.algal.2018.09.004>.

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