Emulsifying properties of ruptured microalgae cells: Barriers to lipid extraction or promising biosurfactants?

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Abstract:

A systematic investigation of the emulsifying properties of ruptured algae cells was performed for the first time. The slurry of ruptured algal cells was separated into different biomass fractions, namely the *cell debris*, the *delipidated debris*, the *serum*, and the *lipid*. The interfacial interactions of these biomass fractions with a nonpolar solvent (e.g. hexane or hexadecane) were characterised using pendant drop tensiometry and interfacial shear rheology. The stability of the different emulsions (formed by the different biomass fractions) was tested using analytical centrifugation. The extracted *lipid* was an excellent surfactant that reduced the interfacial tension, however, it was not effective at stabilising the emulsions. The protein-rich *serum* produced a strong interfacial film that stabilised the emulsions against coalescence during centrifugation. The *cell debris* stabilised the emulsions to a lesser extent by adsorbing to the droplet surface, presumably via interactions with hydrophobic extracellular polymeric substances (EPS). However, neither the *serum* nor the *cell debris* were very effective surfactants, and required the presence of the *lipid* fraction to produce small emulsion droplets. When present together, the components exhibited competitive interfacial adsorption, which influenced emulsion stability. In particular, the interruption of the protein film by the presence of lipid or cell debris reduced the stability of the emulsions. This study provides a new mechanistic understanding of emulsification during wet lipid extraction from microalgae that will be useful for determining strategies to improve solvent recovery. The results also suggest potential for developing effective bioemulsifiers or biosurfactants from fractionated microalgae biomass for commercial application.

Key words

Emulsion; biosurfactant; bioemulsifier; wet lipid extraction; microalgae; interfacial

Graphical Abstract:

Interrupted interfacial film due to competitive adsorption

1. Introduction

Microalgae are seen as a promising source of triacylglycerides (TAG) for the production of biofuels [1]. Recovery of the microalgal lipids must be done directly from a wet concentrated slurry of cells (15-25% solids concentration), to avoid energy-intensive drying, while minimising processing volumes [2, 3]. In a wet algal lipid extraction process, cell rupture is required to allow the solvent to physically recover the intracellular TAG [4, 5]. To minimise the energy required to recycle the solvent, a water-immiscible solvent with a low boiling point (e.g. hexane) is required that can be physically recovered from the wet biomass by centrifugation [3]. The use of water-immiscible solvents results in a biphasic (emulsified) extraction system [5]. It was previously observed that mixing hexane into a slurry of ruptured algal cells formed a tight (stable) emulsion from which it is not possible to recover all of the solvent even with intensive centrifugation [6, 7]. This is currently a major challenge as near-complete recycle of the solvent is required for sustainable biofuel production [4].

Like other microbes [8, 9], microalgae produce an array of biomolecules that could behave as emulsifiers or surfactants. Attached to the exterior of microalgal cells are the so-called extracellular polymeric substances (EPS) [8], that include polysaccharides, proteins, nucleic acids and lipids [10]. Inside the cells there are cytosolic proteins, membrane lipids and other molecules such as DNA [11, 12]. Proteins are excellent emulsifiers, and protein extracts from microalgae have been demonstrated to be no exception [13]. The cell membrane and membranes of the cellular organelles are lipid bilayers comprised mostly of neutral and polar lipids (e.g. phospholipids and glycolipids), which are naturally surface-active [8]. For biofuel applications, microalgae can be induced to accumulate large amounts of TAG [14]. Inside the cells the TAG is stored as lipid droplets that are surrounded by a layer of polar lipids, intercalated with structural hydrophobic proteins [15]. These polar lipids and hydrophobic proteins can potentially continue to stabilize the lipid droplets after cell rupture.

In addition, the ruptured algal cell wall debris [5] may adhere to the hydrophobic surface of the solvent droplets [8], resulting in a Pickering emulsion. The need to process at a high-solids concentration (i.e. 15-25% w/w) means that the slurry is highly viscous [16]. This can stabilize an emulsion kinetically by retarding the creaming and coalescence process.

There is clearly an abundance of potential surfactants and emulsifiers in a slurry of ruptured algae that can stabilize an emulsion, however it is not currently known what roles they play in stabilizing the emulsions and which are the most important (e.g. to perhaps selectively target for removal or degradation). Although emulsions formed by wet algal biomass have been reported [17-20], no systematic investigation has been performed to understand the fundamental contributing factors.

To help develop this understanding, a concentrated slurry of ruptured microalgae cells was separated into major biomass fractions, and the interfacial properties and stability of the emulsions formed with these fractions were characterized. The results from this systematic study were used to develop a mechanistic understanding of the emulsification and demulsification processes in the wet lipid extraction. This understanding is required to develop targeted demulsification strategies to make lipid recovery from microalgal biomass feasible.

2. Materials & Methods

2.1 Microalgae cultivation and harvest

A commercial relevant strain of the marine microalga, *Nannochloropsis salina* [7] was used in this study. To generate the biomass, cultures were grown indoors in 15 L carboys at 20 °C with a light:dark cycle of 14:10 hours. The cultures were grown in a modified f-medium [21] which consists of 255 mg/L NaNO₃, 37 mg/L K₂HPO₄, 9 mg/L ferric citrate, 9 mg/L citric acid, 0.2 mg/L MnCl₂·4H₂O, 0.023 mg/L ZnSO₄·6H₂O, 0.01 mg/L CuSO₄·5H₂O, 0.0084 mg/L Na₂MoO₄·2H₂O, and trace amounts (<1 ppb) of H₂SeO₃ vitamin B12, biotin and thiamine. Gas exchange was delivered via an aquarium air pump (Stellar 380D, Aqua One, China) at a total flow rate of 190 L/h, which was split into 4 air stones. The cultures were harvested into pastes after a 14-day growth period by centrifugation (Separator OTC 2-02-137, GEA Westfalia, Italy) and were stored frozen at -20 °C until use. The solids concentration of the pastes was determined based on the dry weight fractions after oven drying at 60 °C [22].

2.2 Cell rupture via high pressure homogenization

To perform the experiments, the *N. salina* paste (ca. 30% w/w total solids) was diluted to 11-15% w/w total solids using 3% artificial sea water (Coral Pro Salt, Red Sea Fish Pharm LTD, Israel). The slurry was pre-treated by incubating at 37 °C for 24 hours to weaken the cell walls prior to a rupture by high-

pressure homogenisation [7, 22] (Panda 2K NS1001L, GEA Niro Soavi, Italy) using a single pass at 1400 $\pm$ 100 bar.

2.3 Fractionation of the ruptured algal biomass

After cell rupture, the algal slurry was separated into different fractions using centrifugation (Figure 1). The overall slurry of ruptured algal cells is designated as *ruptured total*. Next, a portion of the *ruptured total* was centrifuged at 3000 x g for 10 minutes (Allegra X-30R, fixed-angle rotor F0685, Beckman Coulter, Victoria, Australia). The supernatant obtained was filtered (0.22 μ m Durapore, Millex GC, Cork, Ireland) to remove insoluble particulates. This filtered supernatant is designated as *serum*. The remaining pellet was washed with 3% artificial sea water and re-centrifuged 5-7 times, and finally resuspended to the original volume with 3% artificial sea water. This washed pellet is designated as *cell debris*. Between each centrifugation interval, the samples were mixed on a rotary tube mixer (RSM6, Ratek Instruments, Victoria, Australia) for 1 hour at room temperature.

On a separate portion of the *ruptured total*, n-hexane (AR grade, Ajax Finechem, Victoria, Australia) was added at a 1:1 volume ratio to the wet algal slurry to extract the lipids. The hexane and wet slurry were mixed (RSM6, Ratek Instruments, Victoria, Australia) for 2 hours at room temperature before centrifugation at 9000 x g for 10 minutes. The lipid-rich hexane layer obtained is referred to as *lipid*. For experiments requiring the *lipid* to be dissolved in hexadecane, the lipid-rich hexane was dried under nitrogen [21] and then resuspended in n-hexadecane (99% ReagentPlus, Sigma Aldrich, MO, USA). After decanting the hexane layer, the remaining pellet was repeatedly extracted with fresh hexane (up to 6 cycles) to remove the hydrophobic components. The remaining wet solids from this process was then washed multiple times with 3% artificial seawater to remove the supernatant components. The resulting pellet (excluding the emulsion layer) was retrieved and is designated as *delipidated debris*.

To normalise the concentration of the biomass fractions, the term ‘biomass concentration’ was used to represent the concentration of the isolated component as present in the *ruptured total*. For instance, the *serum* and *cell debris* isolated from an 11% w/w solids concentration (salt-free basis) of the *ruptured total* is described as an 11% w/w biomass concentration. The biomass concentration of the *delipidated debris* was obtained by accounting for the dry weights of the hexane-extracted materials as well as the

serum that were removed. In subsequent experiments where further dilution of the samples was required, the biomass concentration (salt-free) of the biomass fractions was determined by recording the dilution factors rather than measuring the dry weights of the diluted samples.

Figure 1: The separation scheme to fractionate the slurry of ruptured *Nannochloropsis* sp. cells into different biomass fractions. In brief, the whole ruptured-algae slurry was fractionated into different biomass fractions following the different treatment pathways as indicated in colour.

2.3.1 Characterization of the biomass fractions

The total solids concentration of each biomass fraction was measured by oven drying at 60 °C until a consistent dry weight is reached [22]. The dry weight contributed by salt were determined by keeping track of the diluent (3% artificial seawater) added during the experiment. The total nitrogen in the fractionated *serum* was measured by combustion in a CNS elemental analyser (TruMac CNS, Leco Corp., MI, USA) and a nitrogen-to-protein conversion factor of 5.86 was used [23]. The *lipid* was fractionated into neutral lipids, glycolipids and phospholipids using solid phase extraction (SPE) technique [21]. In brief, dried algal lipids were eluted through pre-packed silica columns (SampliQ, Agilent Technologies, Australia) using chloroform, acetone:methanol (9:1, v/v) and methanol.

2.4 Interfacial tension measurement

A pendant drop tensiometer (OCA20, Dataphysics, Germany) was used to determine the interfacial tension between the aqueous and organic phases [24]. n-hexane was used as the organic bulk phase in a 10 mm x 10 mm solvent-resistant cuvette with a PTFE stopper. A drop of the aqueous phase was formed from the top using a gas-tight glass syringe with a stainless steel Luer lock needle (21 gauge). The measurements were performed at an equivalent of 5.0% w/w biomass concentration to prevent

viscosity effects. The densities of the solutions were measured on a calibrated 5 cm³ pycnometer (Blaubrand, Germany). All measurements were performed during day time in a temperature-controlled laboratory (22±2°C).

2.5 Interfacial Shear Rheology

The interfacial shear rheology was performed in a cell (inner diameter of 79.8mm) with a biconical geometry (radius of 68.3 mm with a 5° angle) using an electro-driven rheometer (MCR 702, Anton Paar, Austria). To perform the measurements, 50 mL of the diluted biomass fractions were added into the interfacial rheological cell. The bicone was positioned exactly at the interface using a protocol in the supplied software (Interfacial Gap Sensing app, Anton Paar) and the position visually confirmed. Then 15 mL of n-hexadecane (with or without *lipid*) was gently added to the top along the stem of the bicone so as not to disturb the interface. An extremely small oscillatory shear strain (0.1%) was applied at low frequency (1 rad s⁻¹) and the measured torque converted to a complex interfacial viscosity value based on previously presented principles and calculations [25, 26].

All experiments were conducted at 23°C. For experiments longer than 7 hours, 0.1 wt. % sodium azide was added to the aqueous phase to minimize any potential bacterial activity. A strain amplitude sweep (0.1%-1000% strain) was also performed at the end of the interfacial rheology measurements to check the final breakage strength of the film formed.

2.6 Emulsion stability tests

To determine the emulsion stability, 3 mL of each biomass fraction and 3 mL of n-hexane were added to an airtight and solvent-resistant conical centrifuge tube (15 mL polypropylene tube, Tarsons, Kolkata, India). The contents were mixed at 24 rpm for 2 hours at room temperature on a rotator tube mixer (RSM6, Ratek Instruments, Victoria, Australia). 1.3 g of the emulsified mixtures were sampled and centrifuged using an analytical centrifuge (LUMiFuge®, L.U.M. GmbH, Berlin, Germany) [6]. In the centrifugation tests, a small centrifugal force (30 x g) was applied initially to allow the hexane droplets to cream. After 12 minutes, the centrifugal force was incrementally doubled every 5 minutes until the maximum centrifugal force of the instrument (2330 x g) was reached. As the separated hexane layer is transparent to the near infrared light used by the instrument, and the emulsion layer is opaque, the height

of the emulsion layer was tracked to monitor the phase separation process and to derive the height of the separated hexane layers. The percentage of hexane recovered as a phase separated layer above the emulsion was presented as a function of both the centrifugation time and the centrifugal force.

2.7 Microscopic examination of emulsions

To visualize the emulsified droplets, emulsion samples from section 2.6 were placed between a microscope slide and a cover slip and inspected by bright field microscopy (BX51, Olympus, Tokyo, Japan). For samples with coarse emulsions, the samples were also observed without cover slip to ensure the application of cover slip did not change the droplet size of the emulsion.

2.8 Experimental reproducibility and analysis

All the experiments were performed in at least duplicate. All dry weight measurements of the algal slurries and fractionated biomasses were performed in duplicates. The interfacial tension measurements were performed in duplicate on duplicated samples. The interfacial rheology experiments were performed once for each duplicated biomass fraction sample and the *ruptured total* from two different batches were measured twice each (i.e. four measurements in total). The emulsion stability tests were performed on duplicated samples. The trend was observed to be consistent between the duplicates and a representative set of the data is presented for clarity.

3. Results and Discussion

The emulsifying properties of ruptured *N. salina* cells were investigated by studying the individual fractions separated by centrifugation (Figure 1). The total slurry of ruptured cells (*ruptured total*) was fractionated into *serum*, *cell debris*, *lipid*, and *delipidated debris* components. The mass fraction of each component in the *ruptured total* (on a salt-free basis) was 22 ± 1 % w/w *serum*, 18 ± 1 % w/w *lipid*, and 60 ± 2 % w/w *delipidated debris*. The *cell debris* was essentially the *ruptured total* minus the *serum*, consisting of ruptured and intact cells and some lipids (as confirmed in extraction tests). The *serum* consisted of soluble intracellular components, in particular protein, that comprised 52-57% w/w of the soluble material (based on a nitrogen-to-protein conversion factor of 5.86 [23]). The *lipid* included hydrophobic molecules that partitioned into the nonpolar n-hexane during the first solvent contact [27],

and consisted mainly of neutral lipids (91-93% w/w) and a small amount of polar lipids (5-7% w/w glycolipid and 1-2% w/w phospholipid). The *delipidated debris* included ruptured and intact cells with the nonpolar components removed via repeated hexane extraction.

3.1 Interfacial tension

The interfacial tension is an indirect measurement of the work required to increase the interfacial area [28]. A lower interfacial tension will allow smaller droplets (i.e. tighter emulsions) to be formed, which generally leads to more kinetically-stable dispersions. The interfacial tension was measured using the pendant drop technique, with n-hexane as the bulk phase and seawater as the drop phase. The biomass fractions were then introduced into the appropriate phases (hexane or seawater) and the resulting interfacial tension measured as a function of time (Figure 2).

Figure 2: Interfacial tension of the various biomass fractions in a seawater-hexane system. All experiments were performed by adding the dissolved biomass fractions into either hexane (bulk) or salt water (droplet) phase (3% artificial sea salt). The measurements were performed in duplicates ($n=2$ each batch for a total of two batches) with a standard deviation of < 0.2 mN/m within the batch and < 1.5 mN/m between batches. All the biomass fractions were normalised to $5.0 \pm 0.1\%$ biomass concentration (salt-free), except for the *lipid* which was diluted to an equivalent of 0.4% biomass concentration as appropriate pendant drop images could not be obtained above that concentration. The term ‘biomass concentration’ was used to represent the concentration of the isolated components as present in the *ruptured total*. For example, the *serum* isolated from *ruptured total* of 5.0% w/w salt-free solids concentration is designated as having 5.0% biomass concentration.

To compare the relative surfactant strength of the biomass fractions the pseudo-stable interfacial tension at 1000s is considered. In the absence of any surfactants, the seawater-hexane interface had a high

interfacial tension of 46.5 ± 0.3 mN/m after 1000 sec (Figure 2), close to that of a pure water-hexane system (50.8 mN/m) [29]. The addition of the individual biomass fractions reduced the interfacial tension to varying degrees. To assess the impact of cell rupture on the surfactant activity, both intact (non-homogenized) and ruptured cells were compared. At 1000 sec, the sample with unruptured cells had an interfacial tension of 26.4 ± 0.2 mN/m, much higher than the *ruptured total* at 12.0 ± 0.1 mN/m, showing that the emulsifying capabilities of the slurry were enhanced by the release of the intracellular contents that act as bio-surfactants.

The interfacial tension resulting from the presence of the individual *cell debris* and *serum* fractions were 16.0 ± 0.1 mN/m and 18.6 ± 0.2 mN/m at 1000s, respectively. This shows that both the cell debris and serum contained surface-active components, and that they were more effective in combination (12.0 ± 0.1 mN/m as *ruptured total*) than individually. Even though the *delipidated debris* had the lipids and serum components removed, it was still able to lower the interfacial tension significantly (17.1 ± 0.2 mN/m at 1000s). This suggests that the cell (wall) fragments can adsorb at the interface and reduce the interfacial tension. These results are unexpected as large particles typically do not reduce the interfacial tension significantly. This however can likely be explained by the presence of surface-active components on the particles (e.g. cell debris) that promote the interfacial tension reduction [30].

The *lipid* (added to the hexane phase) reduced the interfacial tension the most (7 ± 0.1 mN/m), resulting in an even lower interfacial tension than the *ruptured total*. This was unexpected considering the *ruptured total* also contained the lipids. In addition, the result was based on the *lipid* at 0.4% biomass concentration instead of 5.0%, as an acceptable pendant drop image could not be obtained above that concentration. This shows that the lipids are the most surface-active compared to any of the other biomass components. The ineffectiveness of the lipid molecules in the aqueous *ruptured total* at lowering the interfacial tension could be due to displacement at the interface by less effective surfactants, or the inability of the lipids to reach the interface due to being trapped within the cell debris.

3.2 Interfacial viscosity

The formation of a pseudo-plastic interfacial film or a high interfacial viscosity is generally a good predictor of a stable emulsion [31]. The presence of such an interfacial film can be detected by

interfacial rheometry, a technique that has been successfully applied to protein- [32], particle- [33] and asphaltene-stabilised [34] emulsions.

In an interfacial shear rheological measurement, it is assumed that the interfacial flow is decoupled from the bulk phases (water or oil phase) and thus the measured effect only originates from the interfacial film region. A more detailed explanation has been provided previously [25, 26]. In our study, the interfacial shear measurements were affected by the bulk phase viscosity at biomass concentration above 7% w/w (Figure 3A). Another limitation of the current system was the sedimentation of the particulate matter (i.e. the *cell debris*) over time. Thus, comparison studies were performed at 3.5% w/w biomass concentration and were limited to <7 hours (Figure 3B).

Figure 3: (A) The progression of the interfacial viscosity (at 0.1% strain and 1 rad s^{-1}) when the measurement was performed with *ruptured total* (7% biomass concentration) and hexadecane. The high initial torque (which was recorded as the interfacial viscosity) was due to the resistant of the bulk phase (i.e. the algal slurry) rather than the interfacial film. As the cell particles sediment, the measured torque dropped to close to that of the seawater-hexadecane interface. (B) The interfacial viscosities as formed by *serum* and *lipid* at 3.5% w/w biomass concentration. The measured values of other biomass fractions (*ruptured total*, *cell debris*, and *delipidated debris*) were close to the torque detection limit of the instruments (not shown), indicating an absence of a rigid continuous interfacial film. .

Under the conditions of this study the *serum*, containing intracellular protein, was the only biomass fraction that was able to form a rigid viscoelastic interfacial film, as indicated by the positive complex interfacial viscosity (Figure 3B). The interfacial film was detectable almost instantaneously upon sample loading and continued to strengthen as it was allowed to age. Although a positive interfacial viscosity was also detected for the *lipid* (Figure 3B), this was likely due to lipid crystallisation and

deposition at the interface, rather than a true interfacial film. This was evidenced by both microscopic and visual inspections (not shown) that revealed a crystalline structure at the interface. During the measurements (Figure 3B), interfacial viscosity would spontaneously decrease after reaching a peak. This behaviour was consistently observed in repeated measurements with the *lipid* samples, although the peak interfacial viscosities varied. Attempts to perform a strain amplitude sweep damaged the microstructure, as evident by an irreversible reduction in the interfacial viscosity. In a wet lipid extraction process it is unlikely that this lipid crystals film would form, as shear is continuously applied.

Interestingly, the *ruptured total* did not generate a measureable interfacial viscosity despite containing the *serum* components. This suggests that the presence of *cell debris* can interrupt the formation of a strong protein film at the interface, despite the apparently synergistic effect of the *cell debris* and *serum* components in lowering the interfacial tension (Figure 2). This is conceptually similar to other studies where the addition of low-molecular weight surfactants into protein-stabilised dispersions were observed to destabilise the emulsions [35].

During a wet lipid extraction process, the neutral lipids from the ruptured algal biomass are expected to partition into the solvent phase. To test if these lipid molecules could interfere with a protein-based interfacial film, the *lipid*, which was primarily neutral lipids, was added to a serum-hexadecane system. It was observed that the interfacial viscosity decreased significantly after the addition of the *lipid* into the hexadecane phase (Figure 4A). An amplitude strain sweep of the serum-hexadecane interface without the lipid addition showed a highly viscoelastic interfacial film (Figure 4B), similar to a protein-stabilized interface [26]. After the lipid addition, the interfacial film was significantly weaker and less elastic, with the G' and G'' cross-over point occurring at a much lower strain. The interfacial shear rheological measurements demonstrate that the simultaneous presence of multiple surfactants and emulsifiers (*cell debris*, *lipid* and *serum*) can result in weaker interfacial films than those formed with protein (*serum*) alone.

Figure 4: (A) Complex interfacial viscosity of a *serum*-hexadecane interface with or without the *lipid*. (B) A strain amplitude sweep was performed for both of the interfaces at the end of the measurements presented in (A) ($t = 24$ h for the *serum* and $t = 32$ h for the *serum-lipid*). The cross over points of G' and G'' in the strain amplitude sweeps are indicated, showing that the *serum*-hexadecane film was much stronger in the absence of *lipid*.

3.3 Emulsion stability

When hexane is mixed into a ruptured-algae slurry, a homogenous oil-in-water emulsion is formed, and the neutral lipids are extracted into the hexane droplets. After centrifugation, four distinct layers are formed: a cell pellet at the bottom, an aqueous supernatant layer, an emulsion layer, and a hexane-lipid top layer [7]. The emulsion layer is recalcitrant to centrifugation, but the solvent must be recovered from this layer to allow full solvent recycling. The emulsion stability is therefore critical to a wet lipid extraction process.

As both the prior measurements (interfacial tension and interfacial shear rheology) required diluted samples, analytical centrifugation (LUMifuge) was used to evaluate the emulsion stability at high biomass concentrations (12.3 ± 0.1 % w/w). At this concentration, all the biomass fractions except the *lipid* (i.e. *ruptured total*, *serum*, *cell debris*, and *delipidated debris*) were able to fully emulsify the hexane. The *lipid* samples instead produced dispersed hexane droplets that phase separated within a few minutes in the absence of mixing. This confirmed that while the *lipid* can act as a surfactant and lower the interfacial tension (Figure 2), it is not an effective emulsifier. Thus, the *lipid* was excluded from the emulsion stability tests. In the tests, a low centrifugal force ($30 \times g$) was applied for the first 12 min to allow the creaming of the hexane droplets, before the centrifugal force was roughly doubled every 5 minutes until reaching $2330 g$ (Figure 5). Previously, we determined that the bulk separation of the

emulsified hexane will not occur unless a critical centrifugal force is exceeded that is sufficient to destabilise the emulsions through droplets-coalescence [6]. This critical centrifugal force, which is seen as a sudden increase in the hexane layer height, was 50 x g for the *delipidated debris*, 200 x g for the *ruptured total*, 500 x g for the *cell debris*, and 1000 x g for the *serum* (Figure 5A). The relatively high stability of the serum-stabilised emulsion is consistent with the presence of a highly viscoelastic proteinaceous interfacial film in the interfacial shear rheology (Figure 4).

Figure 5: The phase separation of hexane from emulsions as monitored by a LUMifuge analytical centrifuge. (A) Hexane separation from emulsions formed by different biomass fractions ($n=4$) at a biomass concentration of $12.3 \pm 0.1\%$ w/w. (B) Hexane separation as a function of solids concentration ($n=2$) for the *ruptured total*. The x-axes indicate the centrifugation time and the centrifugal force applied at different times. The y-axis is the percentage of the separated hexane on a height basis (based on the initial added hexane volume). The centrifugal forces at which major coalescence events occurred are noted in the figures.

The *cell debris* emulsion was more stable than the *ruptured total* emulsion at low centrifugation forces, not exhibiting any hexane separation prior to a catastrophic coalescence event at the critical centrifugal force (500 x g). Even though the *ruptured total* contained *serum* in its composition, the emulsion was not as strong as the emulsion produced by the *serum* alone. These results again suggest that the presence of other surface-active molecules can disrupt the protein film, resulting in a less stable emulsion. The emulsion stability of the biomass fractions also did not appear to correlate with their interfacial tension (Figure 2).

In addition to providing the critical centrifugal force, the LUMifuge tests reveal the amount of the hexane retained in the emulsion. The least stable emulsion, the *delipidated debris*, exhibited near-complete hexane recovery ($98\pm2\%$, $n=4$) at the end of the centrifugation tests (Figure 5A). Despite having different critical centrifugation forces, similar amounts of hexane ($81\pm3\%$, $n=4$) were recovered from both the *cell debris* and *ruptured total* emulsions. Only about $60\pm4\%$ ($n=4$) of the hexane could be recovered from the emulsions produced with the *serum* indicating that even higher forces would be required.

Efficient wet lipid extraction requires processing of concentrated slurries [22]. To investigate the effect of solids concentration on the emulsion stability, tests were performed on emulsions produced with *ruptured total* at 12.3% w/w, 8.8% and 4.4% biomass concentration. The results (Figure 5B) show that as the solids concentration decreased (from 12.3 % to 4.4%), the critical centrifugal force required to coalesce the droplets also decreased (from 200 x g to 75 x g) and the amount of hexane recovered increased. This could be explained by the reduced viscosity and amount of surface-active agents at decreased solids concentrations.

3.4 Qualitative analysis of the emulsion droplets

To evaluate the droplet sizes in emulsions formed with the different biomass fractions (at 12.3% w/w biomass concentration), samples of the emulsions from the stability tests (in Figure 5A) were visualised under a microscope (Figure 6A). The droplet sizes were only assessed qualitatively, as the dispersed hexane droplets were tightly packed and flocculated, preventing an accurate quantitative analysis. Representative images of the emulsions formed by each biomass fraction (Figure 6A) suggest that the droplet sizes correlate to the interfacial tension measurements. In particular, the emulsions containing the *lipid* (i.e. the *cell debris* and the *ruptured total*), which contributed to the lowest interfacial tension, had smaller droplets ($<200\text{ }\mu\text{m}$) than the *delipidated debris* and the *serum* samples which did not contain the lipids (up to $500\text{ }\mu\text{m}$). The droplet size does not appear to relate to the results of interfacial rheology or stability experiments, suggesting that the droplet size has relatively little influence on coalescence. This can also be expected as particles-stabilized emulsions are often observed to be stable to

392 coalescence, despite forming large droplets, as a result of steric hindrance and high energy of particles
 393 detachment [36, 37].

Figure 6: (A) The images are representative of the hexane droplets formed by the *serum*, the *delipidated debris*, the *ruptured total*, and the *cell debris*. (B) The qualitative differences between the hexane droplets from the emulsified *ruptured total* and *cell debris* were assessed after being diluted with seawater and allowed to settle overnight.

399
 400 As both the *ruptured total* and the *cell debris* appeared to be stabilised by particles (Figure 6A), further
 401 investigations were made to test if the emulsions could be destabilised by removing the interface-bound
 402 particles from the emulsified droplets. To test this hypothesis, emulsions were diluted (9-fold) with
 403 seawater, mixed, and allowed to settle overnight. Unexpectedly, an emulsion layer was still present the
 404 next day without a visible hexane layer on top. The hexane droplets in the *cell debris* emulsion were
 405 largely covered by apparently whole cell particles, while the *ruptured total* droplets had some areas

without any visible cell particles attached (Figure 6B). The exposed areas must have been stabilised by other surfactant molecules (e.g. proteins and lipids that are not visible under the microscope). These observations provide further evidence that the cell particles can stabilise the interfaces of the emulsion droplets but can also be displaced by the other surface active molecules.

After washing, the cell particles were still adhered to the surface of the hexane droplets (Figure 6B). This is unexpected given that algal cells are hydrophilic, being natively well-dispersed in water. In addition, even though the *delipidated debris* and the *cell debris* should have both stabilised the emulsion via a Pickering mechanism (i.e. solids-stabilised), the removal of the nonpolar components via hexane extraction seems to have reduced both its emulsifying ability and hexane retention (Figure 6A). One explanation is that the adherence of the cell particles to the hexane droplet was facilitated by the surface-bound EPS on the cells, which were removed during the hexane extraction. Some of these EPS can contain hydrophobic moieties (proteins or lipids) that are typically hidden in a hydrophobic core [10], but can be exposed upon contact with a nonpolar solvent [38].

Prior studies [36, 37, 39] have demonstrated that solid particles, especially those with dual wettability (amphiphilic), can irreversibly adsorb to an oil-water interface due to the high desorption energy requirement. Consistent with this, we have observed that if a low centrifugal force ($\leq 600 \times g$) is used for separating the hexane from the emulsions, only three of the four layers will be formed (namely the hexane layer, the emulsion layer and the aqueous supernatant), with the wet solids pellet (containing solid particles detached from the hexane droplets) being absent. In this case, the low centrifugal force may not be sufficient to overcome the desorption energy barrier and release solid particles from the hexane droplet interface. These results suggest that the hydrophobic components on the cell surfaces (likely the surface-bound EPS) are responsible for the hexane retention after centrifugation (Figure 6A). Thus, these surface-hydrophobic components could be targeted to increase solvent recovery. Another strategy could be to add a low molecular weight surfactant to occupy both the water-oil interface and the hydrophobic cell surfaces, to encourage the release of the cell particles from the solvent interface.

3.5 Mechanisms of emulsification in a nonpolar solvent-based wet algal lipid extraction

Based on this study, a mechanistic understanding of the emulsification and demulsification processes during wet lipid extraction from microalgae can be consolidated (Figure 7). After the algal cells have been ruptured and the intracellular contents released, a nonpolar solvent (e.g. hexane) is added to the ruptured-algae slurry to extract the neutral lipids [4, 5]. The application of shear during the extraction process facilitates the transfer of the *lipids* into the hexane phase. The presence of the *lipid* in the hexane phase decreases the interfacial tension significantly, allowing small hexane droplets to be formed by the shear. In the aqueous phase, the protein in the serum is able to stabilise the emulsified droplet by forming a viscoelastic interfacial film while the cell debris adsorbs to the interface via hydrophobic sites on the cell surface. The presence of multiple surface-active molecules means that there is competitive interfacial adsorption between the lipids, cell particles and proteins, resulting a heterogeneous interfacial film with a weaker emulsion stabilizing capability.

Based on these findings, the recovery of the solvent from the emulsified biomass can be considered as a two-fold problem. First, a critical centrifugal force is needed to destabilize the bulk emulsion by overcoming the interfacial layers, enabling droplet coalescence. This allows up to 80-90% hexane to be recovered as a separate phase. However, to recover the hexane remaining in the recalcitrant emulsion layer, the solids particles have to be detached from the solvent interface. As there is a high energy barrier to detachment, a more effective strategy than centrifugation will be required.

Figure 7: A summary of the emulsifying roles of the surface-active components present in a slurry of ruptured algal cells. Note that the components are not drawn to scale.

3.4 Microalgae as a source of biosurfactants and bioemulsifiers

This study has demonstrated that ruptured microalgae contain a variety of surface-active agents with different characteristics. The fractionation approach taken in this study is a first step towards understanding the emulsifying properties of these surface-active agents. In the future it may be interesting to isolate the individual surface-active molecules for in-depth characterisation. In this regard, microalgae may be considered as a source of biosurfactants and bioemulsifiers, which may have more immediate commercially promise than biofuel production. Industries such as the food, cosmetic and pharmaceutical industries are moving towards substituting synthetic surfactants with naturally derived alternatives [40, 41]. Microalgae can be produced at large scale as a source of surface-active fractions with interesting and distinct properties. For example, the *lipid* is an excellent surfactant that has almost no stabilising effect, whereas the *serum* is highly effective as an emulsifier that is only a moderately effective surfactant and produces relatively coarse emulsions. These distinct and varied properties may allow them to be tailored for specific applications.

4. Conclusions

A systematic investigation of the emulsifying properties of different components of ruptured microalgal cells was performed for the first time. The interfacial tension and the interfacial shear rheology demonstrated that each of the biomass fractions contributed to the stability of emulsions in different ways. The *lipid* acts primarily as a surfactant that lowers the interfacial tension, but does not contribute to emulsion stability. Conversely, the protein-rich *serum* is only moderately effective as a surfactant, but it is able to stabilise the emulsion through the formation of rigid interfacial films. The *cell debris* solids can also help to stabilise the emulsion to a lesser extent by adsorbing at the interface. When multiple components are present there is competitive interfacial adsorption. As such, lipids and cell debris can interrupt the protein film, so that emulsions produced with all the biomass fractions were somewhat easier to break than those produced using solely the protein-rich serum. To ensure a full solvent recovery in a wet lipid extraction process, the detachment of the adsorbed cell particles from the solvent interface appears to be needed. The insights from this study suggest it would be worthwhile investigating the use of individual surface-active agents or combinations as biomass fractions as bioemulsifiers or biosurfactants for commercial applications.

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Declaration of interest

The authors declare no conflict of interest.

Declaration of contributions

S. Law designed and conducted the experimental program, and drafted and revised the manuscript. S. Metthu and M. Ashokkumar assisted in the experimental design and revision of the final manuscript. P. Scales contributed to the conceptual development and interpretation of the research, and revised the final manuscript. G. Martin assisted in the design of the experimental program, data interpretation, and preparation and revision of the manuscript. All authors approved of 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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622 Supplementary Materials

Figure S1: Example of LUMifuge data with an illustration of the centrifuge tube at the top. The positions of the interface between the layers can be determined based on differences in the light transmission % through the centrifuge tube (indicated by the dash lines). As centrifugation progresses, the colour of the traces changes from red to green. The quantity of separated hexane can be determined based on the position of the interfaces.

Figure S2: Comparison of the dynamic interfacial tension for (A) *ruptured total* (B) *serum* and (C) *cell debris* at different biomass concentrations. Interfacial tension isotherms (D) for the biomass fractions were plotted based on the interfacial tension value at 1000 sec for each of the biomass fractions (S.2A - S.2C). The isotherm shows that the *ruptured total* provided a synergistic effect with respect to its combination of *serum* and *cell debris* components, in reducing the interfacial tension more than either of the fractions individually. This synergistic effect is likely because both the cell debris and the serum were able to complement each other at the interface due to their difference in size.

Table S1: Conversion of biomass concentration to actual concentration of different biomass fractions

Experiment	Biomass Concentration (%)	Salt-free concentration of each biomass fraction		
		<i>Serum</i> (mg/mL)	<i>Delipidated Debris</i> (mg/mL)	<i>Lipid</i> (mg/mL)
Figure 2	5.0%	11.0 ± 1.0	30.0 ± 2.0	9.0 ± 1.0
Figure 3A	7.0%	15.3 ± 1.4	41.9 ± 2.8	12.6 ± 1.4
Figure 3B & 4	3.5%	7.7 ± 0.7	20.9 ± 1.4	6.3 ± 0.7
Figure 5A & B	12.3%	27.1 ± 2.5	73.8 ± 4.9	22.1 ± 2.5
Figure 5B	8.8%	19.3 ± 1.8	52.7 ± 3.5	15.8 ± 1.8
Figure 5C	4.4%	9.7 ± 0.9	26.4 ± 1.8	7.9 ± 0.9

Notes:

1. The concentration of each biomass fraction was estimated based on the biomass composition of 22±1% *serum*, 18±1 *lipid* and 60±1 % *delipidated debris* as determined in a separate experiment (i.e. not measured in each experiment).

2. In each experiment, the dry weights of the non-fractionated algal biomass were measured in duplicates, and the amount of salt were tracked by recording the amount of diluent (3% artificial seawater) added.

3. The concentration of *cell debris* (*ruptured total* with the *serum* fraction removed) can be obtained via the sum concentration of *delipidated debris* and *lipid* fractions.

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