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Short Communication

# Algal cell disruption using microbubbles to localize ultrasonic energy

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## HIGHLIGHTS

- Ultrasound with pre-loaded microbubbles was used to enhance algal cell disruption.
- This process requires less than one-fourth the energy of current disruption methods.
- Disruption scales with ultrasound pressure and microbubble concentration.
- Separating bubble formation and growth increases efficiency by localizing energy.
- This process can potentially synergize with dissolved air flotation cell harvesting.

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## ABSTRACT

Microbubbles were added to an algal solution with the goal of improving cell disruption efficiency and the net energy balance for algal biofuel production. Experimental results showed that disruption increases with increasing peak rarefaction ultrasound pressure over the range studied: 1.90 to 3.07 MPa. Additionally, ultrasound cell disruption increased by up to 58% by adding microbubbles, with peak disruption occurring in the range of $10^8$ microbubbles/ml. The localization of energy in space and time provided by the bubbles improve efficiency: energy requirements for such a process were estimated to be one-fourth of the available heat of combustion of algal biomass and one-fifth of currently used cell disruption methods. This increase in energy efficiency could make microbubble enhanced ultrasound viable for bioenergy applications and is expected to integrate well with current cell harvesting methods based upon dissolved air flotation.



## 1. Introduction

Lipid extraction is a key step in algal biofuel production, and disrupting the cell prior to extraction has been shown to improve the recovery of lipids by up to a factor of four (Lee et al., 2012). Several disruption methods are used in labs or full-scale processes, but the energy for disruption of these techniques is typically higher than the energy of combustion of algal biomass (Lee et al., 2013). A survey of disruption techniques suggests energy requirements between $3.3 \times 10^7$ J/kg of dry biomass for hydrodynamic cavitation up to $5.3 \times 10^8$ J/kg for high-pressure homogenizers (HPH) (Lee et al., 2012). Unfortunately, the heat of combustion of algal biomass is only $2.7 \times 10^7$ J/kg (Lee et al., 2012), so current cell disruption techniques typically result in a negative net energy balance in biofuel applications.

However, there is no expectation that the energy is applied efficiently in these devices. For example, when algae are disrupted with an atomic force microscopy (AFM) tip, the specific energy of disruption is only $6.73 \times 10^2$ J/kg, approximately $10^5$ times smaller than the current state-of-the-art hydrodynamic cavitation (Lee et al., 2013). Theoretical estimates based upon individual cell properties also suggest significantly lower specific disruption energy than current processes. One estimate based on the tensile strength of the cell walls suggests a cell disruption energy of $2.26 \times 10^2$ J/kg dry biomass (Lee et al., 2012). A similar estimate based upon anticipated bonding energy in the cell walls is $3.32 \times 10^2$ J/kg (Lee et al., 2012). The energy for cell disruption using the critical tension to rupture a lipid membrane suggests only $1.3 \times 10^{-1}$ J/kg of cell biomass (Krehbiel, 2014).

There are several aspects of current sonication techniques that could potentially increase the energy efficiency substantially.

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Ultrasonic disruption operates with a continuous low-frequency (about 10 kHz) wave (Lee et al., 2010), which is designed to increase the probability of microbubble generation during a low pressure portion of the wave (Bendicho and Lavilla, 2000). The fluid motion due to the bubble and the shock wave accompanying the strong collapse of a microbubble disrupt the cells with shear, heat, or free radical formation (Lee et al., 2012). The dissipation associated with these mechanisms is evidenced by the need to remove heat from sonicators (Balasundaram and Pandit, 2001). A low probability of strong interaction with nearby cells decreases efficiency (Lee et al., 2013).

Significant flexibility would be afforded by separating the bubble loading from the insonification. In this case, the frequency and bubble sizes could be set near resonance to amplify its efficacy. Similarly, the probability of cell-bubble interactions could be specified by changing the concentration of pre-loaded microbubbles. The bubbles have the potential to localize the ultrasonic energy in space, near the algal cells. Their presence, however, also facilitates localization in time. Since the bubble response will be rapid with properly tuned ultrasound, it can then be applied in pulses rather than continuously. This application will further reduce energy required because the ultrasound can be off most of the time. Furthermore, the energy of bubble formation in sonication processes is inefficient (Lee et al., 2012), but bubbles could be generated during a separate stage much more efficiently. For example, the energy for shelled microbubble generation is $6.17 \times 10^6$ J/kg (Krehbiel, 2014).

Ultrasound contrast agents (UCAs) provide the regularity needed to assess the potential of this pre-loading approach. These microbubbles were initially designed to enhance diagnostic ultrasound by increasing the acoustic impedance mismatch. They are made of thin protein or lipid shells with typical diameters between 1 and 10 μm (Stride and Saffari, 2003). It has been established in biomedical applications that when these microbubbles collapse, due to strong acoustic forcing, the resulting pressure gradients and microjets disrupt cellular membranes (Stride and Saffari, 2003). It has been demonstrated that they can be used to transiently disrupt cell membranes so that molecules, such as drug molecules, can be transported into cells (Stride and Saffari, 2003). Here, their potential for total disruption of algal cells is investigated.

The efficacy of bubble action in disrupting cells, whether in biomedical or algal applications, is expected to depend upon the intensity of the ultrasound, the character and dynamics of the bubble, and the relative proximity of the cells to the bubble, though the relative importance of these factors is unclear. The overall objective of this study is to examine the potential of pre-loading with microbubbles to significantly advance the efficiency of ultrasonic algae disruption. Ultrasound contrast agents are used to provide a controlled microbubble geometry and pre-loaded concentration.

## 2. Methods

### 2.1. Algae

*Chlamydomonas rheinhardtii* colonies were cultivated on Petri dishes and then transferred to 250 ml Erlenmeyer flasks with 150 ml TAP medium. They were subsequently grown on a shaker table with continuous lighting ($110 \pm 30$ μmol photons $m^{-2}\,s^{-1}$) at 24 °C. Tests were initiated during exponential growth phase, and a cell count (with hemocytometer) showed $8.9 \times 10^6$ cells/ml. Total suspended solids were 0.76 mg/ml.

### 2.2. Microbubbles

The contrast agent used for these experiments was Definity™ (Lantheus Medical Imaging, N. Billerica, MA, USA). These are lipid-shelled microbubbles with a diameter in the range of 1.1–3.3 μm with a reported mean of 1.98 μm (King and O'Brien, 2011). The experimentally-measured resonant frequency is between 4.0 and 4.5 MHz for 2 μm diameter Definity™ microbubbles (Sun et al., 2005).

### 2.3. Ultrasound setup

The experimental configuration involved flowing the algal solution through a clear vinyl tube with inner diameter 1.6 mm and wall thickness 0.79 mm. A section of the tube in a water bath was insonified by a 0.9 MHz transducer with f-number of 2 (Valpey Fisher, Hopkinton, MA). This frequency provided a beamwidth that covered the cross section of the tube: the −6 dB beamwidth of the pulse is 4.6 mm (Cobbald, 2007). The alignment of the transducer relative to the tube was determined by transmitting a pulse to an air-filled tube; the point of peak amplitude reflection in pulse-echo mode was selected.

Ten-cycle tone bursts were generated at a pulse repetition frequency of 1000 Hz using a pulse-receiver system (RITEC RAM5000, Warwick, RI) providing a duty cycle of 1.1%. Solutions with microzbubble concentrations ($C_b$) between 0 and $15 \times 10^7$ UCAs/ml were prepared by pipetting the appropriate volume of a stock solution of $10^{10}$ UCAs/ml into a 20 ml algae-filled syringe. Uniform distribution of the UCAs in the syringe was ensured by gently rolling the syringe vertically and horizontally for 30 s prior to each test. The solution was visually well-mixed and the rise times based on Stokes drag of the microbubbles (16 min) far exceeded the testing time. Peak acoustic rarefaction pressures ($P_r$) of 0, 1.90, 2.38, 2.83, and 3.07 MPa were measured with a polyvinylidene fluoride hydrophone following established procedures (Raum and O'Brien, 1997).

For each test, 3.0 ml of the algae and microbubble solution was pumped through the tube at a rate of 40 ml/hr (0.67 ml/min) with a syringe pump, though only the final 1.5 ml were collected to ensure that only samples treated with the selected test conditions were collected. For each test condition, four 180 μl samples were analyzed in a microplate reader, and each test condition was repeated three to ten times.

### 2.4. SYTOX fluorescence diagnostic

To quantify cell viability, SYTOX green fluorescent probe was used (Molecular Probes Inc. Eugene, OR, USA) because it has been shown to correlate well with disruption and extractable lipids (Roth et al., 1997; Sheng et al., 2011). To do this, the 5 mM solution was diluted to 10 μM with deionized water, and 20 μl were mixed with 180 μl of treated algal solution in each well of a 96-well plate. This produced a final 1.0 μM SYTOX concentration in accordance with recommendations for eukaryotes (Life Technologies, 2006). The well plate and cover were placed in a microplate reader (Infinite 200 series, Tecan Group Ltd. Männedorf, Switzerland) and the samples were shaken for 10 s with a 1 mm orbital amplitude and then excited with 488 nm light. Fluorescent emission was measured at 534 nm with gain set at 80%. This measurement was repeated for 30 min. Maximum fluorescence values during the 30 min are reported.

To correlate SYTOX fluorescence with disruption of *C. rheinhardtii*, the algal cells were made to be permeable with 70% isopropyl alcohol as proposed by Roth et al. (1997) with the modification that the sterile dilute medium was replaced with deionized water. The disrupted cells were then combined with untreated cells, and three separate calibration samples with 0%, 20%, 40%, 60%, 80%, and 100% of disrupted cells were analyzed. The measured relationship between fraction disrupted ($\Delta d_f$) and maximum fluorescence relative to an untreated control ($F$) was well fitted by

$$\Delta d_f = 0.338\ln(F) \quad (1)$$

with an $R^2$ value of 0.975.

## 3. Results and discussion

### 3.1. Experimental results

Microscope images of the cells in the presence of microbubbles with and without ultrasound treatment showed the effect of the ultrasound. Without treatment the cells appeared intact, and the contrast agent bubbles were clearly visible. After sonication, the cells were visibly damaged, confirming that cell disruption occurred with this setup. No UCAs were observed in treated samples, confirming their destruction by sonication. Reported results suggest that $P_r$ of 2 MPa caused postexcitation collapse of Definity™ contrast agents when insonified with a three-cycle tone burst of 0.9 MHz (King and O'Brien, 2011), indicating that complete bubble disruption would occur under the present operating conditions.

Measured fluorescence intensities relative to a control without ultrasound treatment were converted to percent change in disrupted algae $\Delta d_f$ using (1). Fig. 1 shows that samples without contrast agents showed no significant increase in disruption, indicating that insonification without microbubbles did not disrupt the cells. For concentrations above $0.1 \times 10^7$ UCAs/ml, cell disruption increases with microbubble concentration and ultrasound intensity up to $C_b = 12.5 \times 10^7$ UCAs/ml for most testing pressures. However, the maximum $\Delta d_f$ is 58% at $C_b = 10 \times 10^7$ UCAs/ml and $P_r = 3.07$ MPa.

For all the pressures tested, disruption was lower for $C_b = 15 \times 10^7$ UCAs/ml than $C_b = 12.5 \times 10^7$ UCAs/ml, suggesting an inhibitory effect at these highest concentrations. This would be consistent with attenuation of the sound waves by scattering from the bubbles which would shield a portion of the bubbles in the tube from the incident sound waves.

At low $C_b$, there is an unintuitive but repeatable larger $\Delta d_f$ at $1.0 \times 10^7$ than $2.5 \times 10^7$ UCAs/ml. This was confirmed with multiple samples tested on multiple days. It is interesting to note that this maximum occurs near the cell concentration for which there are equal numbers of microbubbles and cells, though any particular importance of this is as yet unclear. It might reflect a balance of low shielding, due to low microbubble concentration, but still sufficiently close average bubble-cell spacing to yield significant disruption. In general, this suggests that different factors may dominate algal disruption at various microbubble concentrations.

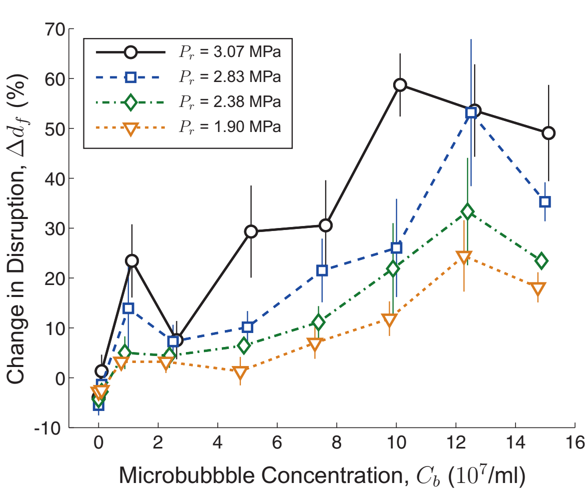


**Fig. 1.** Change in cell disruption as a function of microbubble concentration at different ultrasound peak rarefactional pressures. The mean value and standard error of three to ten samples are plotted for each pressure and concentration.

### 3.2. Energy relative to other cell disruption methods

The acoustic energy required to disrupt algae in the current setup was estimated by knowing the pressure profile of the wave and shows a specific disruption of 43 J/kg of dry algal biomass (Krehbiel, 2014). It should be recognized that this value only accounts for the acoustic energy, not the entire energy of the tone burst or the electrical energy to create the pulse, though contributions from such factors are expected to be small. Modern transducers have reported efficiencies above 95% (Beijing Ultrasonic, 2013), so the electrical energy to create the acoustic pulse approximately equals the acoustic energy.

Fig. 2 shows that this disruption energy compares favorably with other disruption-only estimates. It is somewhat less than the estimates based upon stress analysis of cell walls and AFM measurements but significantly more than estimates of energy required to disrupt lipid membranes. To compare with full disruption energy requirements of established processes, the energy budget needs to include an estimate of the energy required to form the contrast agents: $6.17 \times 10^6$ J/kg of dry biomass (Krehbiel, 2014). If microbubbles are alternatively generated in dissolved air flotation (DAF), used to harvest algae, $7.1 \times 10^6$ J/kg of dry algal biomass is required (Wikramanayake et al., 2012). Even with the microbubble generation energy included, the proposed cell disruption method is estimated to use less than one fourth of the available heat of combustion of algal biomass and approximately one-fifth of the specific energy required for hydrodynamic cavitation, $3.3 \times 10^7$ J/kg (Halim et al., 2012), which is the lowest of the currently used commercial methods.

If bubble formation occurred with DAF, the energy required for cell harvesting could be shared with the energy for cell disruption and product extraction. Other disruption methods require additional cell harvesting energy, while integrating DAF with the present disruption technique would not. Thus, the energy benefit of

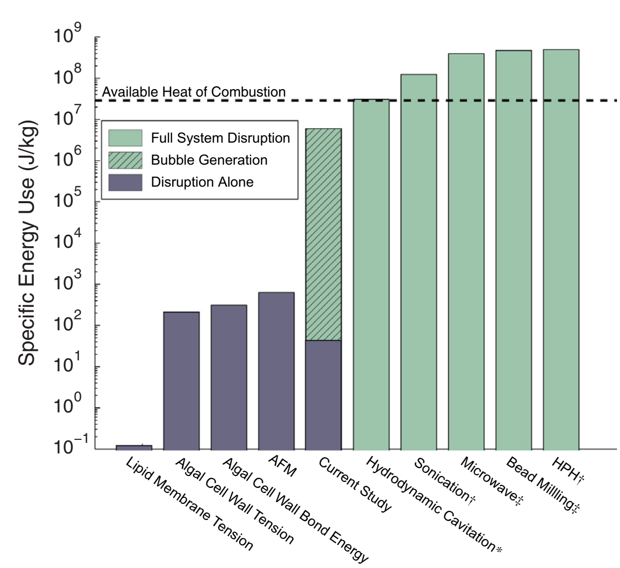


**Fig. 2.** Specific energy requirements of several different algal cell disruption techniques. The thick dashed line is the typical heat of combustion of algal biomass (Lee et al., 2013). Citations: * (Balasundaram and Pandit, 2001), † (Halim et al., 2012), ‡ (Lee et al., 2010).

this method would be even greater than the difference between bubble generation and full system disruption.

The apparent energy advantage of the current approach relative to other insonification methods can be explained by several aspects of the respective methods. The energy is localized near the cell by increasing the concentration of microbubbles and decreasing the average cell-to-bubble spacing. Pulsing the wave decreases energy use and reduces heating. Finally, the frequency of the acoustic wave is close to the bubble resonant frequency to improve efficiency, since less energy is required if the bubble responds strongly to the incident sound waves.

It is somewhat surprising that the present method seems to require less energy than the direct application of force via an AFM tip, and several aspects of the respective methods might yield this difference. For one, the microbubble disruption is more rapid and dynamic. The cells experience forces that vary over microseconds, whereas the AFM experiment was so slow it might be approximated as quasistatic. Even a pure mechanical response of a viscoelastic body varies considerably based upon the character of the force, even before any rate-dependent biological response might become important. The algal species are also different, so no close agreement would even be expected even for AFM. The cell wall of *Tetraselmis suecica* in the AFM experiments is mainly 3-deoxy-manno-2-octulosonic acid, which is different from the glycoprotein cell wall of the present *Chlamydomonas* (Hoek, 1995; Lee et al., 2013).

It is consistent that the current technique is between energy anticipated by models based on algal membrane tension and bond energy and the energy expected by the critical lipid membrane tension. The theoretical models assume that damage to 25% of the cell membrane is required to disrupt the cells, whereas the present method only requires sufficient damage to allow SYTOX to enter the cell and fluoresce. The disruption energy of typical lipid bilayers is provided as a lower-bound reference for the energy required for cell disruption. However, the cell walls of algae are stronger than typical lipid bilayers, so the ultrasound energy requirement is expected to be above this lower bound.

## 4. Conclusions

Ultrasound exposure of algae with pre-loaded microbubbles shows significant cell membrane disruption, up to 58%. Disruption increases with both peak negative ultrasound pressure and bubble concentration. The disruption energy is at least four times lower than current scaled-up methods and comparable to theoretical estimates. Its energy advantage derives from flexibility afforded in separating bubble generation from insonification and the localization of energy in space and time. This process could significantly improve the energy balance for production of algal biofuels, especially if it can be synergistically combined with dissolved air flotation to efficiently harvest and disrupt algal cells for lipid extraction.

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