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A mechanistic study of algal cell disruption and its effect on lipid recovery by solvent extraction

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ABSTRACT

The effect of cell rupture on subsequent lipid recovery by solvent extraction was assessed by performing extractions on *Chlorella* sp. suspensions that were ruptured to varying extents by high pressure homogenization. Descriptions of lipid recovery mechanisms were supported by scanning electron microscopy (SEM) images of the damaged cells, with the extent of cell rupture quantified by cell counting. Lipid recovery by monophasic solvent extraction was found to be a two-stage process. Intracellular lipids from ruptured cells were immediately recovered. Lipids from unruptured cells were recovered very slowly over hours, which can be best explained by diffusion across the cell wall, driven by a concentration gradient formed upon permeation of solvents into the cells. Biphasic solvent extraction required complete rupture of the cells to enable the necessary physical contact between the immiscible solvent and the lipids. Lipid recovery from ruptured cells was 100-fold higher than that from unruptured cells, owing to the impermeability of the cell walls to the immiscible solvent. Biphasic solvent extraction selectively recovered non-polar triacylglycerides, while monophasic solvent extraction recovered all lipid types. This study demonstrates and explains the need for complete cell rupture in the recovery of intracellular lipids from microalgae using solvents, and provides the first clear description of the underlying mechanisms.

1. Introduction

Many species of microalgae (hereinafter algae) are described as oleaginous due to their ability to produce substantial amounts of intracellular lipids that can be converted to biodiesel [1–3] and other high value products such as omega-3 fatty acids [4–6]. Biodiesel production from lipids, in particular triacylglycerides (TAGs), extracted from algae is a promising source of sustainable biofuel due to the high areal productivities for biomass generation without competing for arable land or fresh water with existing agriculture [7]. However, the development of processes for the conversion of algal biomass to biodiesel to achieve cost efficiencies that rival petroleum-based fuels is an ongoing challenge that demands in-depth understanding of both algal biology and process engineering [8]. In particular, the economic production of algal biodiesel requires that value be derived from all biomass components [9,10], and the development of a highly efficient and effective technology for the recovery of lipids from algal biomass. In addition to the efficient recovery of TAGs, the ability to recover other lipids may be of importance in producing higher value products such as omega-3 fatty acids that may be the primary product in a small-scale algal operation, or a low-volume co-product that can improve the overall economics of larger-scale biofuel production processes.

Processes for extracting TAGs from conventional vegetable oil feedstocks such as soybean and oil-seed crops are well-established, often involving an organic solvent such as hexane used to extract the non-polar TAGs from the dry and permeable feedstock material [11]. However, application of these methods to algal biomass is not viable due to differences in the material properties of unicellular algal biomass that lead to complications in processing. In contrast to conventional TAG feedstocks which have low water content, algae are cultivated as dilute aqueous suspensions, and even after harvesting and concentrating into a paste, considerable amounts of intra- and intercellular water remain. The development of a solvent extraction system that is effective in suspensions of up to 80 wt.% water is highly desirable as mechanical dewatering is not sufficient to remove inter- and intracellular water beyond this point [12] and the addition of a drying step would require sufficient energy to render the overall process uneconomical [13]. For a solvent extraction process to be effective in an aqueous environment, the extracting solvent system must be able to overcome the polar barrier formed by the water [14].

In addition to the difficulties caused by the presence of water, the lipids in algal biomass are contained within rigid cell walls forming a barrier to solvent penetration. It has been suggested that rupturing

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the cell walls prior to solvent contact can greatly improve the resulting lipid yields [15,16]; however the mechanisms of solvent extraction and the effects of cell rupture have yet to be investigated. Cell rupture can be achieved by a range of techniques including mechanical methods such as bead milling, ultrasonication and high pressure homogenization, and non-mechanical methods involving chemical and enzymatic treatments [17,18]. High pressure homogenization has been the most widely used process-scale disruption unit operation [19] and is the most suitable for large scale processing of microalgae due to its scalability, continuous operation and the ability to process wet biomass [16]. The cell walls of some of the most industrially promising algae such as *Chlorella* sp. and *Nannochloropsis* sp. have been shown to be resilient towards mechanical rupture [20]. However, when combined with a pretreatment step, high pressure homogenization has been shown to be highly effective in processing wet algal concentrate of up to 25 w/w% solids as a precursor to lipid recovery without incurring excessive energy demands [16].

An increase in lipid yield by solvent extraction on algal suspensions has been shown to result from the application of various methods of cell rupture including high pressure homogenization [16], ultrasonication [15,21], microwave heating [15,22], bead-beating and osmotic shock [15], providing indirect evidence that cell rupture can improve lipid recovery. However, in these studies cell rupture was not quantified and the relationship between cell rupture and lipid extraction has not been established. The mechanisms of the apparent improvement are also yet to be investigated and the role of cell rupture has not yet been properly described. The process of cell rupture and quantification of the extent of rupture are complicated [23], and establishing a relation between cell rupture and solvent extraction therefore requires a detailed investigation. Hypothesized mechanisms of solvent extraction from microalgae have been proposed [14,24], however these were not directly based on experimental data and do not appear to adequately explain the role of cell rupture or the possible differences between monophasic and biphasic solvent systems.

Monophasic solvent systems are those in which the solvents are miscible with water and which often involve polar/non-polar solvent mixtures such as chloroform/methanol or hexane/isopropanol [24]. These solvents are typically able to recover both neutral (e.g. TAGs) and polar (e.g. glyco- and phospholipids) lipids and are therefore of interest in recovering lipids both for analytical purposes [25] and for smaller-scale industrial extractions targeting higher value products. Biphasic solvent systems, such as hexane in water, are immiscible in water, and in the case of algal lipid extraction, selective towards TAGs and avoid co-extraction of the polar lipid fractions [16]. The conventional feedstock for biodiesel is the TAGs, and the polar lipids are in fact a contaminant in biodiesel production, hence the ability to selectively extract neutral lipids is an advantage for this application. While extraction efficiency is important for applications involving both monophasic and biphasic solvent systems, it is absolutely essential for the economic and energetic viability of large-scale biofuel production. In this regard, extraction efficiency includes minimising the contact time between the solvent and the biomass and minimising the cost and energy input of recovering or replacing the solvent after contact. Despite the critical importance of solvent extraction for lipid recovery from microalgae, little is known about the mechanisms of either monophasic or biphasic solvent systems, the differences between the two, and the role and importance of cell rupture on improving extraction efficiency.

In this study, a detailed examination into the mechanisms of lipid recovery from disrupted algal cells by extraction with both monophasic C/M/W and biphasic hexane-in-water solvent is undertaken. *Chlorella* sp. was chosen to be the representative microalga in this study due to its ability to accumulate high levels of lipids [26]. Quantification of the extent of cell rupture was performed by cell counting [20], with supporting electron microscopy analysis allowing a more detailed examination into cell rupture. While it is clear that cell disruption has a positive effect on lipid recovery, the extent to which this occurs and the underlying mechanism that drives the mass transfer of intracellular lipids into the solvent phase are still not clear. Here we provide conclusive evidence of the need for complete cell rupture and for the first time provide a clear picture of the underlying mechanisms of solvent extraction from microalgae.

2. Materials and methods

2.1. Microalgal strain

A wild strain of *Chlorella* sp. isolated locally from the banks of Cooper Creek, Innamincka in South Australia was used in this study. The culture was maintained in flasks with a flat surface of 25 cm^2 (Corning Incorporated, Corning, NY) at 20 °C under a continuous low photon flux intensity of 6 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by white fluorescent lights. Subcultures were prepared every 4 weeks. The culture was maintained in a modified "f-medium" with nutrients and trace elements [27] in synthetic seawater, with compositions as previously described [26].

2.2. Microalgal cultivation

Microalgal biomass was generated in 5 L bioreactors (Sartorius BIOSTAT® Aplus) with the aforementioned growth media. Cultures were grown at 21 ± 2 °C under an average light intensity of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ in a 12:12 light/dark photoperiod. Mixing was achieved by a continuous aeration of filtered atmospheric air and a constant impeller speed of 150 rpm. Routine microscopic inspections were made to ensure a monoculture is maintained. Cultures in the bioreactor were halved and refilled with fresh medium when the biomass concentration reached 1.0–1.5 $g \cdot L^{-1}$. Cell density was monitored by cell dry weight and turbidity measurements. All experiments in this study were performed on cultures harvested during the exponential growth phase, with the exception of the lipid fractionation experiment (data presented in Figs. 6 and 7). To induce lipid accumulation, cells were incubated under nitrogen-limiting conditions and harvested after 10 days as previously described [26]; lipid formation was monitored microscopically. Microalgal biomass was harvested and dewatered by centrifugation at 4000 *g* for 15 min at 20 °C. The resulting pellet was resuspended in filtered synthetic seawater to avoid osmotic shock.

2.3. Cell disruption

A GEA Panda 2K NS1001L bench top high pressure homogenizer (GEA Niro Soavi, Parma, Italy) equipped with a cell disruption valve (Re+ valve) was used for cell disruption. Microalgal suspensions were passed through the homogenizer at pressures ranging from 250 to 1400 bar. Processed suspensions were recovered for subsequent analyses. All analyses were performed within 3 h of disruption.

Quantification of cell rupture was performed by cell counting due to its accuracy and reproducibility [23,28]. The extent of cell disruption was assessed by the number of intact cells remaining after homogenization using a Neubauer improved haemocytometer (Laboroptik Ltd., Lancing, United Kingdom) with a 100 μm chamber depth. All imaging was performed using an Olympus BX51 light microscope with a DP72 digital camera attachment (Olympus, Mt. Waverley, VIC, Australia). Cell counts were normalized between the control sample (unhomogenized cell count) and zero.

2.4. Lipid extraction

A modified Bligh and Dyer method [25] was used to represent monophasic solvent extraction systems. Chloroform and methanol were added to the homogenized sample to form a 1:2:0.8 v/v/v monophasic suspension which was continuously mixed at room temperature for (i) 20 min (see Figs. 1B and 6) and (ii) 48 h with aliquots