

Lipid releasing characteristics of microalgae species through continuous ultrasonication

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ABSTRACT

In this study, the lipid releasing characteristics of several microalgae species through continuous ultrasonication was examined. Two marine microalgae species, *Tetraselmis suecica* and *Nannochloropsis* sp., and one freshwater species, *Chlorella* sp. were ultrasonicated directly after cultivation. The cell disruption efficiency and lipids releasing pattern from microalgae cells were measured under various ultrasonication conditions. It was found that cell disruption efficiency correlates well with ultrasonication energy consumption despite the ultrasonication conditions. Lipids in *Chlorella* sp. that has rigid cell walls were released to the aqueous phase after cell disruption. *Tetraselmis suecica* and *Nannochloropsis* sp. that have flexible cell membranes tend to coil up and retain the membrane lipids after disruption. Continuous ultrasonication can be a potential method to release the lipids in rigid walled microalgae species without expensive dewatering steps.

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1. Introduction

The lipids present in microalgae can be classified mainly into non-polar and polar lipids. For example, phospholipids, glycolipids and betaine lipids are polar, while acylglycerols, sterols, and free fatty acids are non-polar lipids. The polar lipids and sterols are important structural components of cell membranes and intracellular thylakoid membranes. The thylakoid membranes are mainly composed of the 4 glycerolipids of which monogalactosyl diacylglycerol (MGDG) and digalactosyl diacyl glycerol (DGDG) are abundant, and these lipids contain high amounts of polyunsaturated fatty acids (PUFA) and palmitic acid (C16:0). Triacylglycerols (TAGs) are non-polar storage lipids, which are found inside the cells. The storage lipids (TAGs) contain a much lower percentage of PUFA, but are made up of saturated and mono unsaturated fatty acids. (Harwood, 2004; Guschina and Harwood, 2009).

For efficient extraction of lipids in microalgae cells, different cell disruption techniques have been explored. Various methods such as osmotic shock, microwave treatment, autoclave, bead beating and ultrasonication were studied. However, there is no agreement in the most efficient methods among the literature. For example, one study found the microwave treatment to be the most efficient for lipid extraction (Lee et al., 2010). On the other hand, another study reported ultrasonication to be the most efficient among the same cell disruption methods (Prabakaran and Ravindran, 2011). One other study comparing 9 different cell disruption methods found that grinding with liquid nitrogen gave the highest lipid content, followed by enzymatic lysis and microwave treatment (Zheng et al., 2011). Among the methods used for cell disruption, ultrasonication has gained a lot of attention due to the high scalability from an industrial point of view. When ultrasound is applied, microbubbles are formed in the liquid. The bubbles oscillate and grow when ultrasonic energy is absorbed. As the bubbles reach a critical size, the cavitation of the bubbles can generate substantial shear forces and disrupt the surrounding cells (Mason, 1990). The temperature inside the bubbles

can be as high as 5000K and a pressure of 2000 atm. It has been demonstrated that ultrasound is effective in the extraction process in a number of applications (Moulton and Wang, 1982; Kim and Zayas, 1989). For lipid extraction from microalgae, it was found that the optimum conditions were 1000 W of ultrasonic power, 1800 s extraction time and a microalgae concentration of 5 dry weight percent (Adam et al., 2012).

For all the lipid extraction methods, dewatering of the microalgae is necessary because the presence of water decreases the rate and efficiency of the transesterification process (Kusdiana and Saka, 2004). In a life cycle analysis done on algae biodiesel production, it was found that 89% of the total energy input goes into the dewatering of algal biomass. For every 1000 MJ (or 24 kg) of algae biodiesel produced, the dewatering step alone uses 3292 and 6194 MJ of energy when the separation method is filter press and centrifugation respectively (Sander and Murthy, 2010).

The aim of the present study is to explore the feasibility of lipid recovery directly from wet microalgae culture without the use of solvents and suitable for industrial applications. More specifically, the lipid releasing characteristics of several commonly used marine microalgae (*Tetraselmis suecica* and *Nannochloropsis* sp.) and freshwater microalgae (*Chlorella* sp.) under continuous ultrasonication without prior water removal were investigated. The effects of ultrasound on the cell disruption and lipid extraction profile under various operating conditions were also studied.

2. Materials and methods

2.1 Microalgae and culture media

Three microalgae species were utilized in the study. *Tetraselmis suecica* (Culture Collection of Algae and Protozoa CCAP® 66/4TM) and *Nannochloropsis* sp. (Culture Collection of Algae and Protozoa CCAP® 211/46TM) are marine species and *Chlorella* sp.

(American Type Culture Collection, ATCC® 14854TM) is a freshwater species. *Tetraselmis suecica* was cultivated in Walne medium (Walne, 1970) using artificial seawater of 45 g/L salinity and enriched with 10 g/L sodium bicarbonate as carbon source. The *Nannochloropsis* strain was cultivated using seawater, which had been filtered to 20 microns before UV treatment. The culture was kept nutrient replete with F/2 medium and CO₂ gas as the carbon source. *Chlorella* sp. was cultivated using modified R-medium reported in our previous study (Chen et al., 2011).

2.2 Photobioreactor setup and cultivation procedure

The cultivation system consists of a photobioreactor and two light panels as described in our previous study (Chen et al., 2011). Microalgae from agar plates was first transferred to 250 mL conical flasks and incubated in the shaker (Spectra Teknik 200B) for a week and then the algae were cultured in 5 L airlift photobioreactors with mixture gas (air and CO₂) containing 2% (v/v) CO₂ under 25 °C and 100 μmol/m²·s. The total flow rate was set at 0.25 vvm. The microalgae culture was harvested when the cell concentration reaches 2 g/L. The cell growth was monitored by cell counting using a cell count chamber hemocytometer (Fisher USA) and an optical microscope (Fisher).

2.3 Fatty acid analysis

Fatty acid analysis was performed using the *in situ* direct transesterification reaction (Lewis et al., 2000). The freeze-dried microalgae was mixed with 3.4 ml methanol (99.8+% Sigma Aldrich), 4 ml chloroform (99.8+% Sigma Aldrich) 0.6 mL H₂SO₄ (91% Sigma Aldrich) and 0.5 ml of 10 g/L internal standard (heptadecanoic acid Sigma Aldrich). Transesterification reaction was performed in 80°C water bath for 1 hour and cooled down to

room temperature. 2 ml of deionised water was added to separate the organic phase and inorganic phase. The bottom layer was collected for gas chromatograph analysis.

The gas chromatograph (GC) (GC 6890N, Agilent, USA) was installed with Agilent HP-88 capillary column (Agilent, USA). The inlet temperature and the flame ionization detector (FID) temperature were set at 260°C and 275°C, respectively. The column inlet pressure was kept constant at 120 kPa and helium carrier gas was set at a flow rate of 44.3 ml/min. Upon sample injection, the oven temperature was held at 50°C for 1 minute, then raised to 150 at the rate of 10°C/min and held for 2 minutes, then raised to 200°C for 10 minutes at the rate of 8°C/min and finally raised to 240°C at the rate of 20°C/min and held for 8 minutes. Supelco 37 Component FAME mix (Sigma Aldrich, Singapore) was run as the external standard. The measurements were performed in triplicate.

2.4 Continuous ultrasonication of microalgae

The wet culture from the photobioreactor was ultrasonicated directly without prior dewatering. 700 mL of wet culture at a microalgae concentration of 2 g/L were used in each experiment. The ultrasonication system (UIP 1000HD, Hielscher, Germany) consists of an ultrasonic processor (20 kHz, 1000 W) and a sonotrode (BS2d18, Hielscher, Germany) housed inside a flow cell 100 mL in volume, a water cooling jacket for the flow cell, a recirculation tank for feed and processed sample storage, and a centrifugal pump for sample circulation. Wet microalgae sample was initially poured into the recirculation tank. Ultrasonicated samples for characterization are collected at the flow cell exit before returning to the recirculation tank. The biomass after ultrasonication were harvested by centrifugation and subsequently freeze-dried for the analysis of fatty acids retaining in the ultrasonicated biomass.

2.5 Cell disruption efficiency

One important evaluation of ultrasonication performance is the cell disruption efficiency, η . The cell disruption efficiency can be calculated by:

$$\eta = \frac{C_0 - C_f}{C_0} \quad (1)$$

where C_0 is the initial cell concentration before ultrasonication and C_f is the cell concentration of the ultrasonicated sample. The cell concentration was determined by cell counting using a cell count chamber hemocytometer (Fisher USA) and an optical microscope (Fisher). The cell disruption efficiency will be plotted against the energy consumption of the ultrasonication process per unit volume of the cell culture. The energy consumption is calculated by multiplying the ultrasound power to the processing time.

2.6 Scanning Electron Microscopy

The morphology of freeze-dried microalgae before and after ultrasonication was characterized by scanning electron microscope (SEM) (JSM-5600, JEOL, Tokyo, Japan). Dried sample was dispersed onto a carbon tape that is attached to a metal stub. Before examining the sample under SEM, platinum has to be coated on the particle surface under an argon atmosphere (JFC-1600, JEOL, Auto Fine Coater, Tokyo, Japan) with a current of 20 mA for 60 s. SEM images taken at various locations of the carbon tape were used for analysis of the cell structures.

1. Results and discussion

3.1 Effect of ultrasonication on cell disruption

Effects of ultrasonication on cell disruption efficiency were investigated for three parameters, namely ultrasound power, recirculation rate and microalgae cell concentration. Both *Tetraselmis suecica* and *Chlorella* sp. were used for comparison between marine and

freshwater species. Cultures of *Tetraselmis suecica* and *Chlorella* sp. were ultrasonicated at three power levels, 500 W, 750 W and 1000 W. It can be seen in Fig. 1(a) that the cell disruption efficiency increases sharply initially and plateaus as the cell disruption efficiency is approaching 100% for both *Tetraselmis suecica* and *Chlorella* sp. As the cells are disrupted, the cell concentration decreases. The ultrasonication process becomes less energy effective at low cell concentrations (Adam et al., 2012). *Tetraselmis suecica* is found to have significantly higher cell disruption efficiency upon ultrasonication compared to *Chlorella* sp. It requires about 0.110 kW-hr/L of ultrasound energy to achieve 99% cell disruption for *Tetraselmis suecica* while *Chlorella* sp. requires more than 3.5 kW-hr/L to achieve a mere 85% cell disruption. This is possibly because freshwater *Chlorella* sp. has a very rigid three layered cell wall structure (Dodge, 1973). In fact, cell walls of *Chlorella* sp. were found to comprise chitin which can form a strong hydrogen bonding matrix with the neighboring acetyl amine groups (Kapaun and Reisser, 1995). In addition, it has been reported that a decrease in cell diameter reduces the cell disruption efficiency of ultrasonication (Allinger, 1975). *Chlorella* sp. cells have an average diameter of about 2 μ m while *Tetraselmis suecica* cells are almost 10 μ m in diameter. Therefore, the smaller cell diameter of *Chlorella* sp. further contributes to the reduction in cell disruption efficiency compared to *Tetraselmis suecica*.

Fig. 1(a) also shows that the cell disruption efficiencies at different ultrasound powers essentially fell onto the same curve. It indicates that ultrasound power has negligible impact on the cell disruption efficiency for both *Tetraselmis suecica* and *Chlorella* sp. in the range of ultrasound power studied. It is reasonable to consider that ultrasound energy has negligible attenuation in the liquid phase and it is only “consumed” when in contact with foreign objects such as the microalgae cells. It is reported in the literature that ultrasound power has relatively low effect on the oil yields in solvent extraction methods (Cravotto et al., 2008;

Singh and Gu, 2010; Adam et al., 2012). However an increase in ultrasound power can reduce the operating time required to achieve the same cell disruption efficiency.

Fig. 1(b) shows the cell disruption efficiency under different recirculation rates. Three different recirculation rates were tested for *Chlorella* sp. sample and 2 recirculation rates were used for *Tetraselmis suecica*. There is negligible difference in cell disruption efficiencies among the recirculation rates tested. An increase in recirculation rate appears to have a slight improvement on the cell disruption efficiency. It is possibly because a high recirculation rate allows the cells to pass through the ultrasonication flow cell more frequently and provides more uniform exposure to the ultrasound energy.

Effects of cell concentration on cell disruption efficiency were studied on *Chlorella* sp. from a concentration range of 0.07 g/L to 12.2 g/L. To aid the interpretation of the result, data fitting was performed using the cell disruption model (Sauer et al., 1989):

$$\ln\left(\frac{1}{1-\eta}\right) = kE^a \quad (2)$$

where η is the cell disruption efficiency, k is the rate constant, and a is the exponent for energy consumption. The fitted curves showed good match with the experimental data in all cases (R^2 was between 0.93 and 0.98). As shown in Fig. 1(c), the cell disruption efficiency increases with an increase in cell concentration up to 6.84 g/L. An increase in cell concentration improves the cell disruption efficiency possibly due to lower loss of ultrasonic power on the disrupted cells. Ultrasonication has also been used over higher concentrations of 12.2 g/L. No noticeable change is observed compared with a concentration of 6.84 g/L in terms of cell disruption efficiency. This is due to the fact that the presence of highly concentrated cells increases the apparent liquid phase viscosity and subsequently reduces the efficiency of ultrasonic cavitation for cell disruption (Mason, 1990).

3.2 Comparison of fatty acid compositions with literature

Figs. 2(a)-(c) show the fatty acid compositions of *Tetraselmis suecica*, *Nannochloropsis* sp. and *Chlorella* sp. in comparison with the respective fatty acid compositions reported in the literature. It can be seen in Fig. 2 that there is considerable variation among the fatty acid compositions reported in the literature even for the same species. However, it can still be seen that the most abundant components were relatively consistent. For example, as shown in Fig. 2(a), C16:0 was consistently the most abundant component among the different *Tetraselmis* species reported. Fig. 2(c) also indicates that C16:0 and C18:2 were the most abundant components in *Chlorella* sp. On the other hand, the most abundant fatty acids in *Nannochloropsis* species in Fig. 2(b) show more variations. In one case, C16:0 and C16:1 were the most abundant. In another case, C20:5 can be the most abundant. Despite the differences among the specific strain, the inconsistency in the fatty acid compositions can be derived from the variation of the growth conditions and harvesting time. The fatty acid compositions are largely influenced by the light availability and the specific growth phase at which the algae are harvested. For example, more neutral lipids such as triacylglycerols (C16:0, C18:0) are produced when saturating light conditions were provided. When light is limiting, more galactolipids that contain polyunsaturated fatty acids such as C20:4 and C20:5 are produced (Sukenik et al., 1989). In addition, the fatty acid composition in the cell changes with the growth phase. The amount of eicosapentaenoic acid (EPA) was found to reduce in the late exponential phase or stationary phase compared to the early exponential phase (Huerlimann et al., 2010). All the cultures in this study were harvested in the stationary phase. Therefore the amount of EPA is low compared to some of the literature reported values.

3.3 Effect of ultrasonication on lipid release

The presence of water reduces the efficiency of the transesterification reaction (Kusdiana and Saka, 2004). As a result, the lipids released during the ultrasonication were investigated

based on the lipids retaining in the dried biomass residual. The biomass residual comprises of the cell debris of the disrupted cells. The difference in fatty acid compositions between the pre-ultrasonicated sample and the ultrasonicated sample can indicate the lipids released to the liquid phase. Figs. 3(a)-(c) show the change in the fatty acid compositions in the biomass residual with respect to ultrasonication energy consumption for *Tetraselmis suecica*, *Nannochloropsis* sp. and *Chlorella* sp., respectively. It can be seen that the amount of fatty acids in the biomass residual decreases with an increase in the ultrasonication energy consumption for all three species. While each species started with vastly different fatty acid compositions, the effects of ultrasonication on the release pattern of lipids were consistent. Take *Tetraselmis suecica* as an example, Fig. 3(a) shows that storage lipids including saturated lipids (C16:0) and mono-unsaturated lipids (C16:1 and C18:1) were released rapidly upon ultrasonication. The poly-unsaturated lipids (C18:2n6, C18:3n3) are a part of the polar membrane lipids while the saturated lipid C16:0 is also the construction DGDG glycolipid in thylakoid membranes (Harwood, 2004; Guschina and Harwood, 2009). It is more difficult to release the membrane lipids and therefore a slower or negligible release through ultrasonication is observed compared to the storage lipids.

A comparison of the lipid release pattern in Fig. 3 with the cell disruption pattern in Fig. 1 indicates that the relationship between cell disruption and lipid release pattern is different for different microalgae species. For *Tetraselmis suecica*, while the cell disruption efficiency reached almost a constant beyond 0.110 kW-hr/L of ultrasonication energy, the released lipids continued to increase up to 0.8 kW-hr/L. *Chlorella* sp., on the other hand, has a more positive correlation between the cell disruption efficiency and lipid release percentage. Both cell disruption efficiency and lipid release percentage reached about 75% at ultrasonication energy of 0.8 kW-hr/L. It is possibly because *Chlorella* sp. has a rigid polysaccharide cell wall (Kapaun and Reisser, 1995) and the lipids inside the cell walls of *Chlorella* sp. can be

released upon cell disruption. However, the lipids inside the cell membranes of *Tetraselmis suecica* are more difficult to be released even after the membrane is disrupted. In addition to the different lipid releasing characteristics, ultrasonication also exhibits different disruption characteristics among the three microalgae species. Upon ultrasonication, the damaged *Tetraselmis suecica* cell membrane coiled into rod-shaped structures, which explains the membrane lipid retention characteristics observed. Similarly, the damaged *Nannochloropsis* sp. cell membrane formed small pockets and retained the membrane lipids. In contrast, ultrasonication completely shattered the *Chlorella* sp. cells. Both the storage lipids and the lipids within the rigid polysaccharide cell walls can then be released into the aqueous phase. Therefore, it is anticipated that the use of ultrasonication can be a promising scalable method for releasing lipids in microalgae species consisting of rigid cell walls. Subsequent recovery or utilization of the lipids from the aqueous phase can then be applied with the absence of solvents.

Conclusions

Continuous ultrasonication was found to be effective in releasing the lipids from *Chlorella* sp. that has rigid cell walls. Flexible cell membranes of *Tetraselmis suecica* and *Nannochloropsis* sp. tend to coil up and retain the membrane lipids after disruption. Operating conditions of ultrasonication have negligible effect on cell disruption efficiencies when energy consumption is considered. Continuous ultrasonication can be a potential method to release the lipids in rigid walled microalgae species without expensive dewatering steps. Subsequent recovery or direct utilization of the lipids from the aqueous phase can be applied to eliminate the use of solvent in traditional extraction methods.

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Figure captions

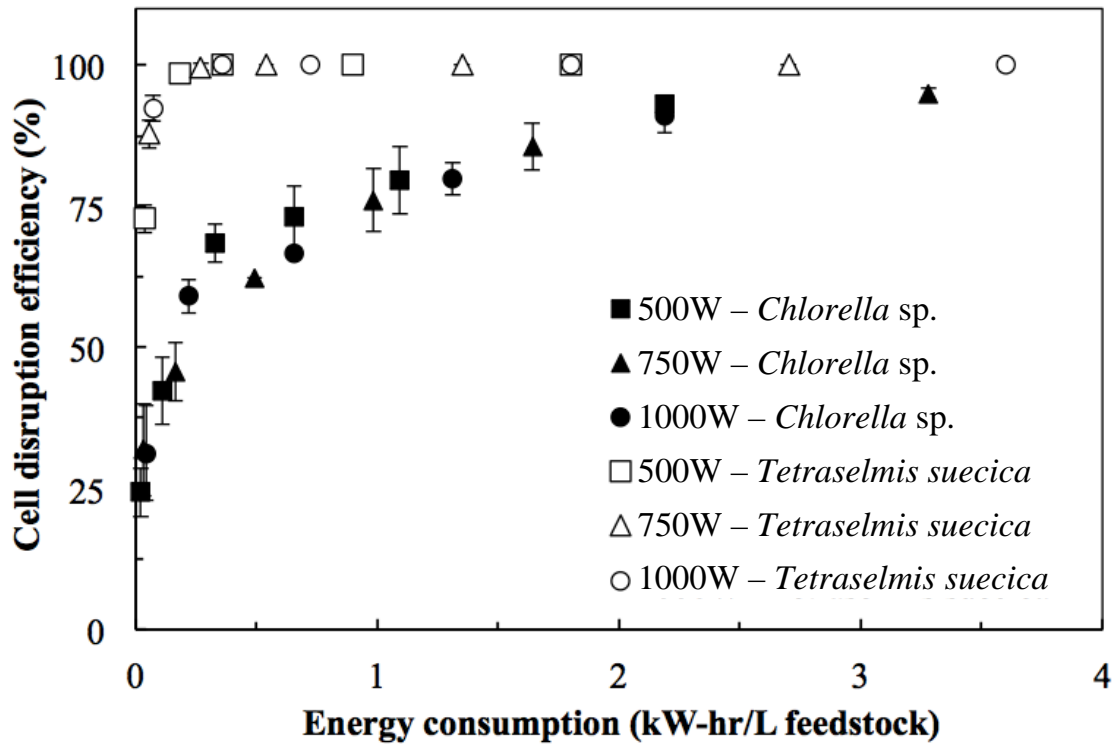
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Fig. 2. Comparison of fatty acid composition among literature reports and current study, (a) *Tetraselmis suecica*, (b) *Nannochloropsis* sp., (c) *Chlorella* sp.

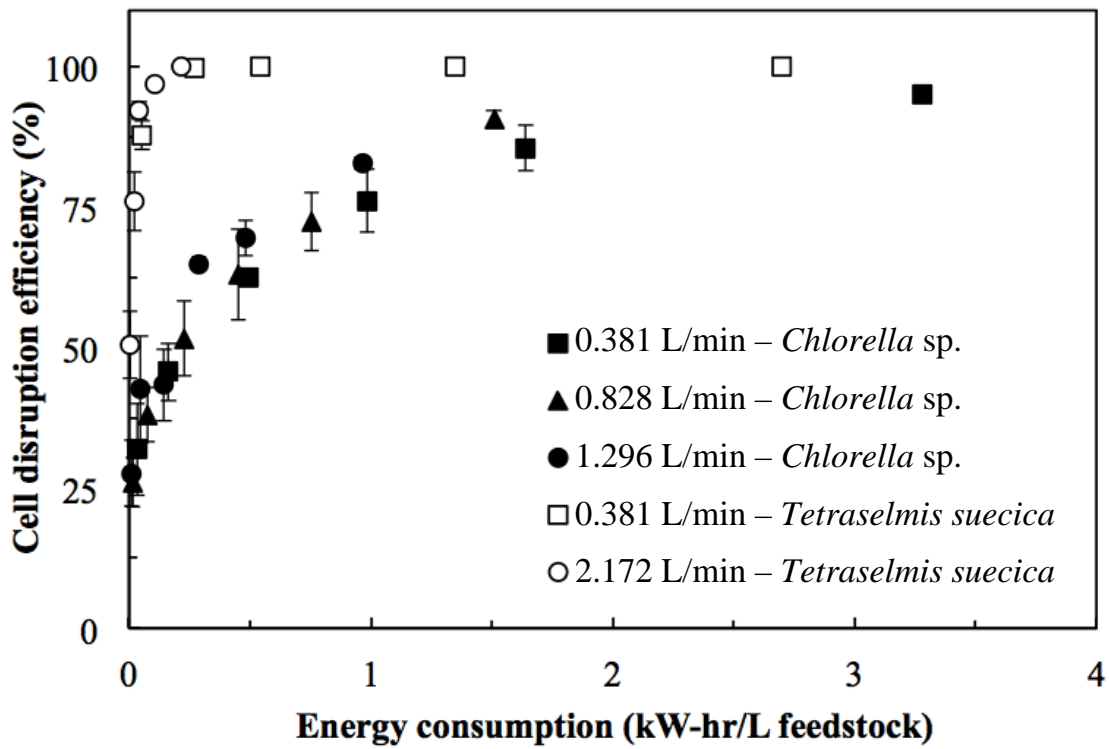
Fig. 3. Effect of ultrasonication on fatty acids retained in biomass, (a) *Tetraselmis suecica*, (b) *Nannochloropsis* sp., (c) *Chlorella* sp.

Fig. 4. SEM images of freeze-dried microalgae (i) before ultrasonication and (ii) after ultrasonication, (a) *Tetraselmis suecica*, (b) *Nannochloropsis* sp., (c) *Chlorella* sp.

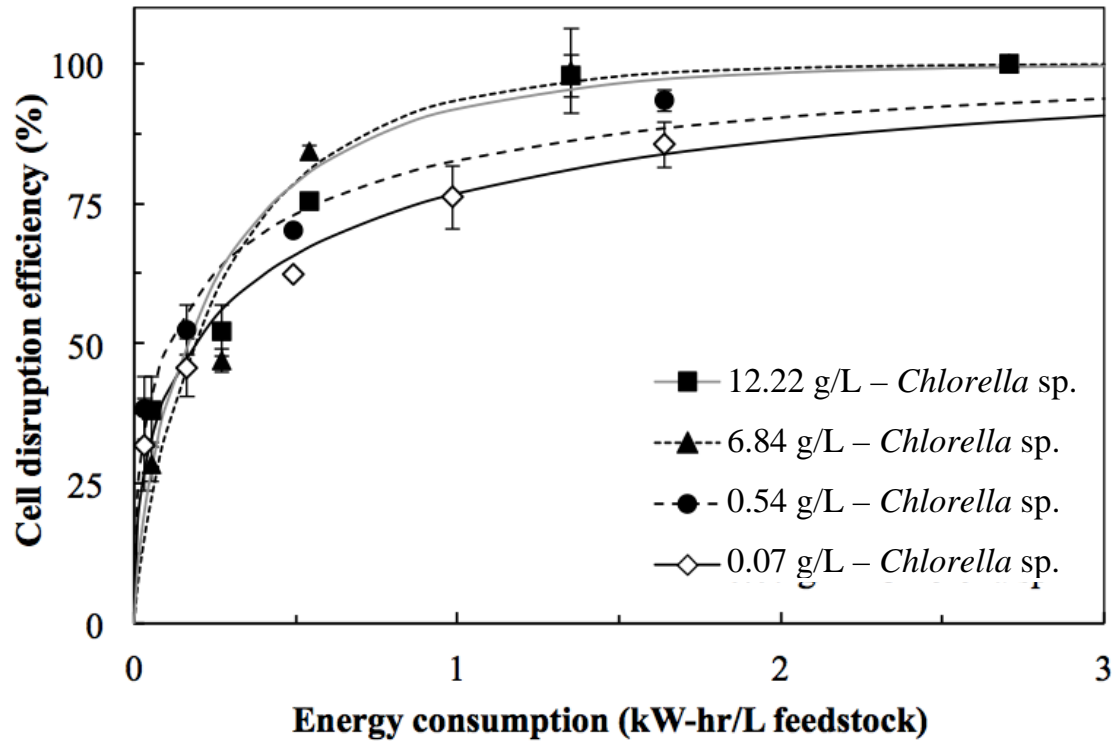
Figures



(a)

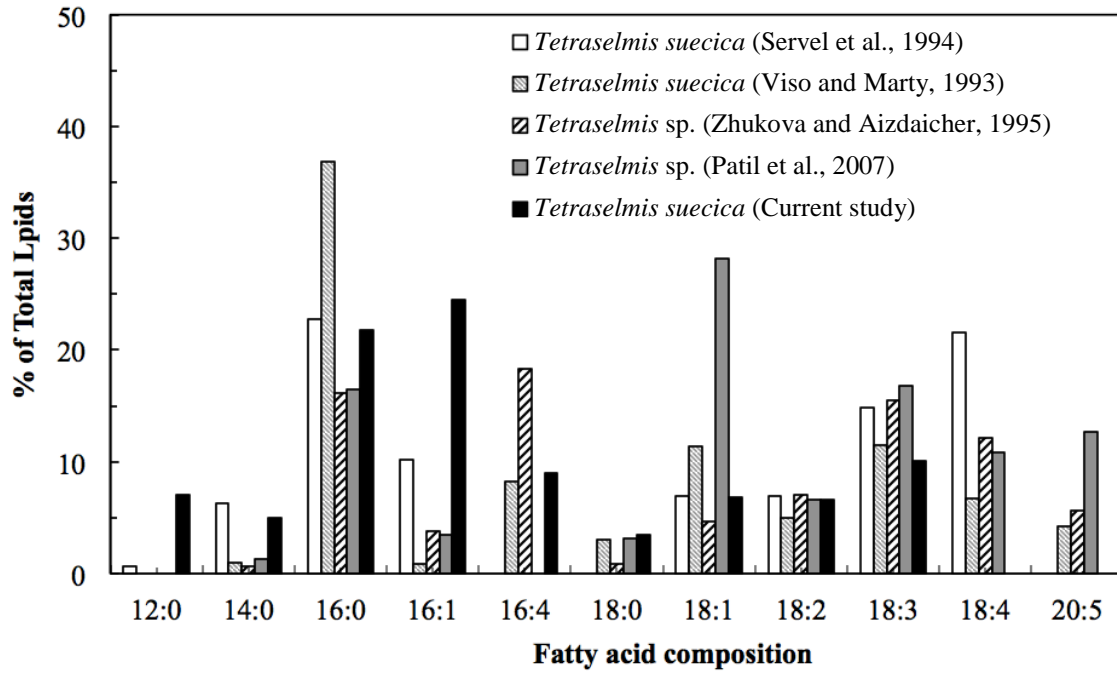


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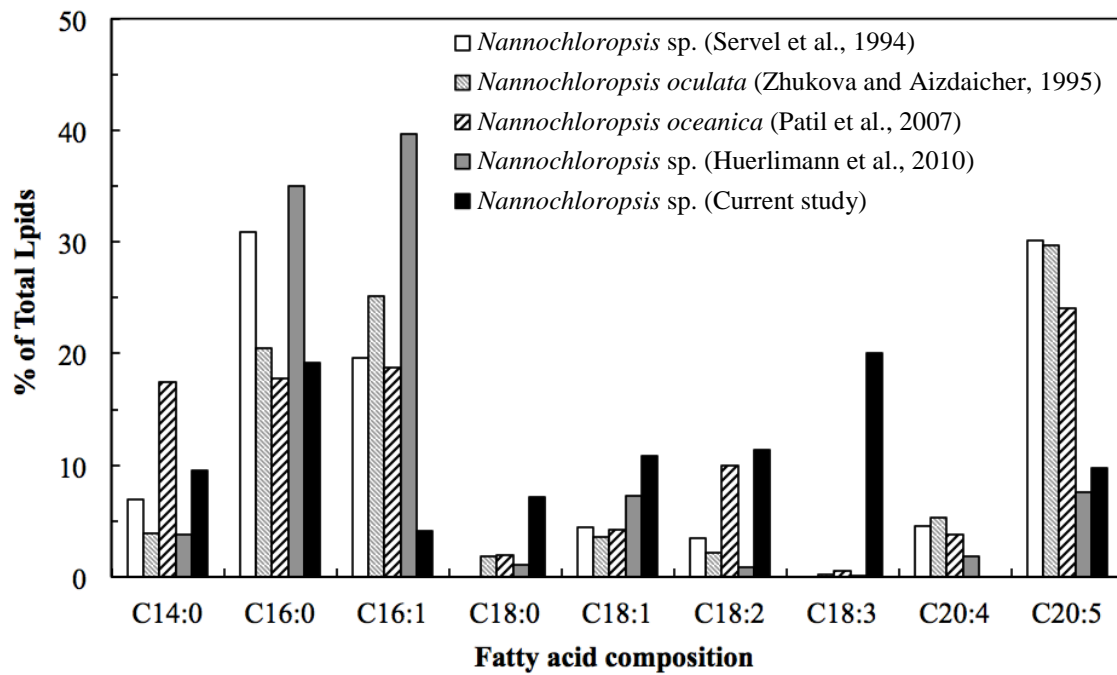


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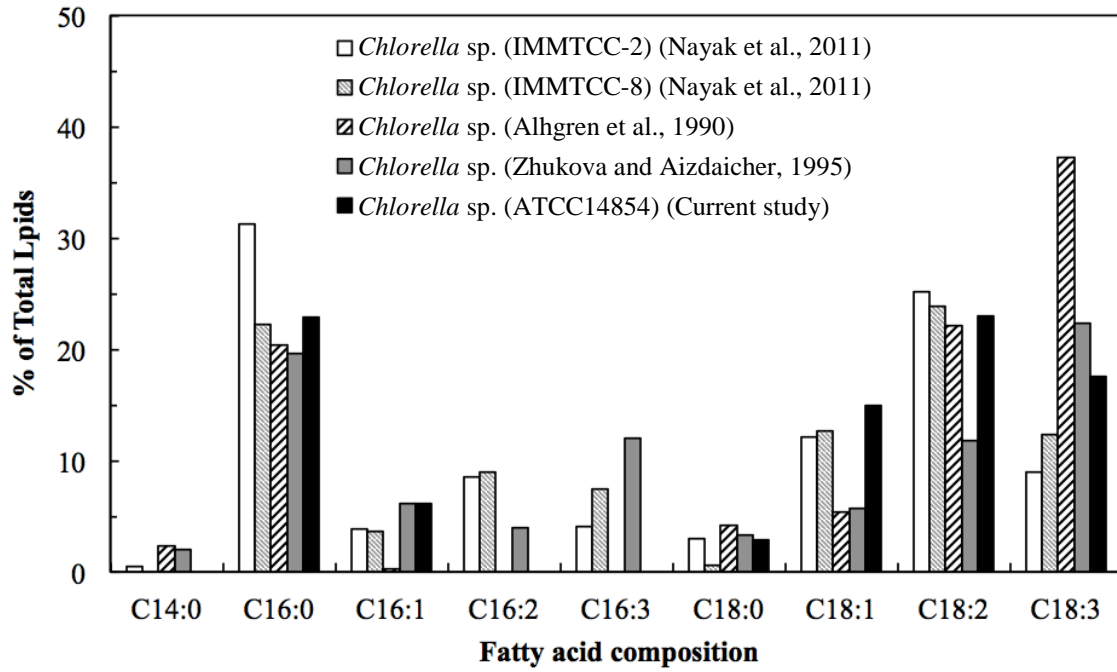
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(a)

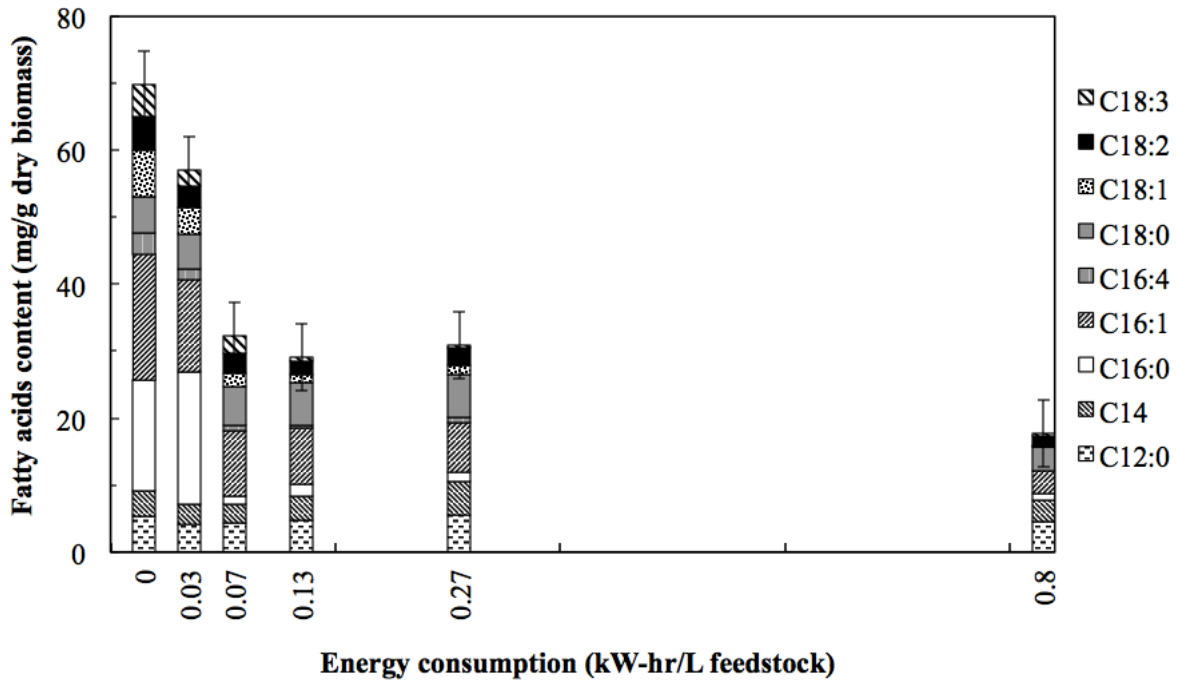


(b)

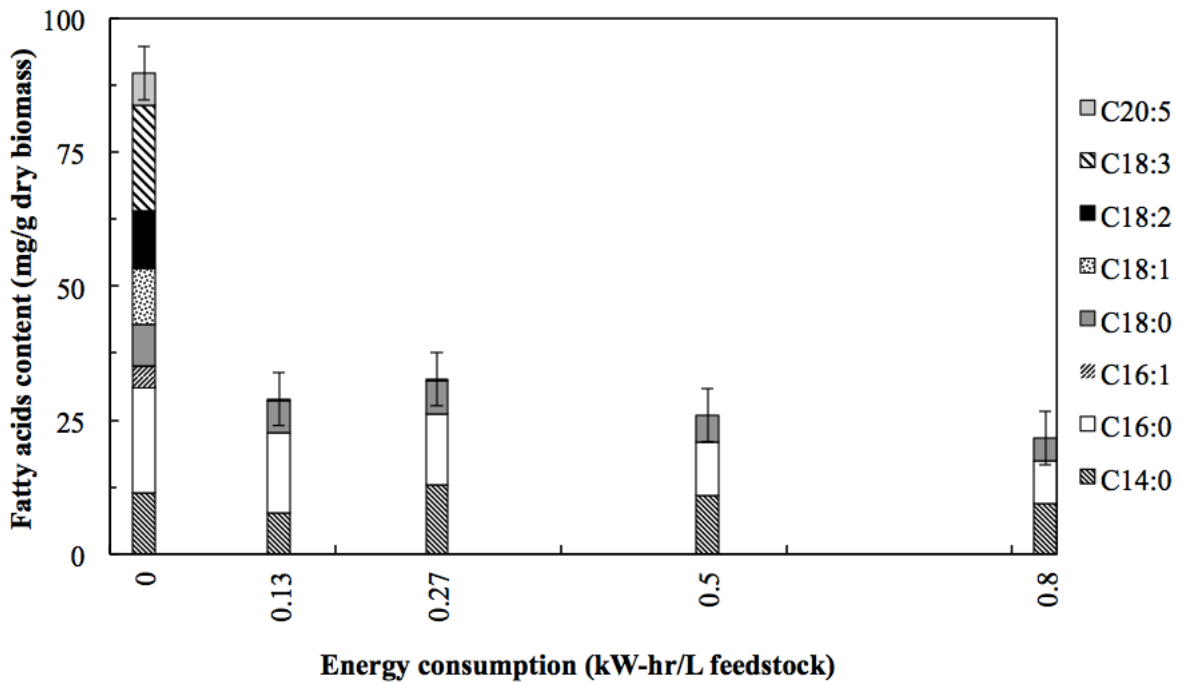


(c)

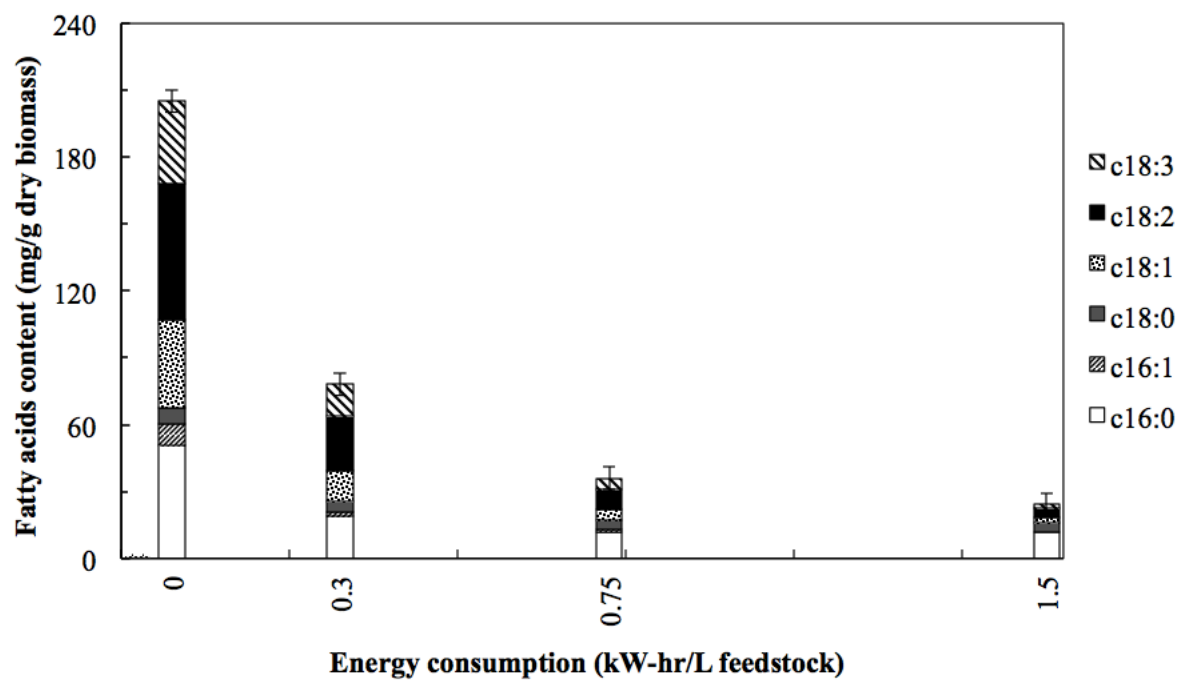
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(a)



(b)



(c)

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