Abstract
The industrial extraction of lipids from microalgae is hampered in part by the energy intensive nature of extraction procedures, and the cost and toxicity of the solvents used. Both liquid carbon dioxide (\(\text{CO}_2\)) and mixtures of methanol and \(\text{CO}_2\) are shown to be viable alternatives for the extraction of lipids from wet microalgae. Both the rate of extraction and the total yield of extract was found to be dependent on the microalgae concentration and stirring. With higher concentrations of microalgae, the extraction yield decreased, likely as a result of the reduced interaction between the algae and the \(\text{CO}_2\). On the other hand through the use of mixed solvent systems of methanol \(\text{CO}_2\) it was possible to achieve higher extractions however, to reach similar extraction with mixed solvents systems it was found that very high volumes of methanol was required. Using mixtures of methanol and \(\text{CO}_2\) it is possible to achieve greater yields, however high volumes of methanol are required.

Key words: Microalgae, liquid \(\text{CO}_2\), \(\text{CO}_2\) expanded methanol, green chemistry, renewable resources

Introduction
The extraction of bio-oil (lipids) from microalgae has been examined as a promising source of biofuels with limited success.\(^1\),\(^2\) The main barrier to implementation has been the high costs associated with processing; particularly harvesting and drying the microalgal biomass, as well as removal (via distillation) or reuse of the solvents employed in the extraction process.\(^1\),\(^2\) Despite these techno-economic challenges, microalgae are still considered a potentially viable source of lipids for the production of biodiesel.

Although the costs of the extraction phase of lipid recovery have been shown to be less than those associated of the drying and dewatering phase, distillation of solvents used in the extraction process is energy intensive, and the traditional solvents used also present environmental concerns.\(^3\),\(^4\) A number of traditional extraction processes make use of flammable and/or chlorinated solvents leading to increased environmental, health, and safety issues and higher costs associated with the recovery, removal or recycling of the solvent(s).\(^3\),\(^5\)-\(^9\)

Improved lipid extraction methods that avoid drying algae and/or solvent removal from algal slurries have also been shown to be effective.\(^1\),\(^10\)-\(^12\) Supercritical carbon dioxide (\(\text{scCO}_2\)) is non-toxic, has excellent mass transfer rates, does not require distillation to produce a solvent-free extract, and has been applied to extract lipids from algal slurries.\(^11\)-\(^14\) While \(\text{CO}_2\) offers the same advantages as \(\text{scCO}_2\) with respect to its toxicity, flammability and recoverability, it requires lower operational pressures and temperatures than \(\text{scCO}_2\), which could potentially translate to lower operational costs.\(^7\),\(^15\) Studies by Paudel et al. (2015)\(^7\) and Chen et al. (2013)\(^16\) showed that \(\text{ICO}_2\) is a viable solvent for the extraction of lipids from microalgae. Chen et al. (2013)\(^16\) found that the yield of lipids extracted using \(\text{ICO}_2\) from freeze dried microalgae, compared to traditional solvent extraction systems, was dependent on the pressure, with 20.5 % extraction (total extract) on the basis of dry microalgae mass at 10 MPa increasing to 60.6 % at 20 MPa.\(^16\) Paudel et al. (2015)\(^7\) showed that the low polarity of \(\text{ICO}_2\) could lead to higher selectivity for neutral lipids compared to traditional solvents using freeze dried microalgae.\(^7\)

However, it is noted that \(\text{scCO}_2\) and \(\text{ICO}_2\) require high pressures and result in comparatively low yields. It is possible reduce these issues through the use of mixed solvents and/or gas expanded solvents such as \(\text{CO}_2\) expanded methanol (\(\text{cxMeOH}\)).\(^7\),\(^17\) Methanol is a good choice as a co-solvent, if one is needed, as it is used in the subsequent transesterification step in bio-oil processing.\(^7\),\(^17\)

It was noted by Yang et al. (2015)\(^18\) using \(\text{scCO}_2\) expanded methanol it is possible to extract up to 84.8 % of total extractable lipids from freeze dried microalgae within 30 min. In addition on the use of ethanol as an extraction solvent the yield of microalgae extract was found to decrease by comparison with \(\text{CO}_2\) expanded methanol. It is shown that increasing temperature of the extraction system results in decreased bio-oil yield. Conversely, increased pressure results in increased bio-oil extraction.\(^18\)
Extraction of lipids from wet microalgae rather than dried microalgae could decrease the process energy costs significantly. Xu et al. (2011) noted that the use of wet microalgae reduced the process energy consumption to only 12% of that required in the thermal drying process, although the energy cost associated with the extraction process tended to increase.

Using scCO₂, Halim et al. (2011) showed that scCO₂ extractions of both dry (>95 wt%) and wet microalgae (30 wt%) resulted in similar lipid yields from Chlorococcum sp., producing up to 5.75% (based on the dry mass of microalgae) after 1 h in a flow-through reactor. This demonstrates that the presence of water in the microalgae did not impede the extraction of lipids. However, the use of IČO₂ for the extraction of lipids from wet microalgae (0.078 - 17.1 wt%) has yet to be explored in terms of the effects of microalgal slurry concentration on extraction efficiency.

This study investigates the effect of microalgae concentration on the rate and yield of extraction to gain a better understanding of the processes occurring within the system. To achieve increased yield of extract, mixtures of methanol/CO₂ were explored in the extraction of wet Chlorella vulgaris, with respect to the effect of variation in the amount of methanol in the system. The selectivity for the extraction of neutral lipids (NL) and free fatty acids (FFA) from microalgae was also assessed for the IČO2 and mixed CO₂ methanol systems.

Experimental Methods

Materials

Microalgae

The microalgal Chlorella vulgaris (UTEX, B1803) was grown in a 23 L glass carboy in modified Bold’s basal medium under continuous illumination using Orphek Atlantik V2.1 (WiFi disabled) Aquarium LED lighting system as described by Lee et al. (2015). The culture was continuously aerated with filtered (0.45 µm) air to provide adequate mixing of the microalgal suspension as described by Lee et al. (2015). A subset sample (10 L) was collected from the bulk sample following 2 weeks of growth at a concentration of 0.78 g/L (by dry mass), and the sample was centrifuged to a concentration of 17.1 wt%.

Chemicals

Methanol (Fischer scientific; 99.8 % purity) was used as the collection solvent for materials vented from the backpressure regulator. Gas used in the experiments was obtained from Praxair (CO₂ liquid tank with dip tube purity 5.0).

Methods

Extraction using IČO₂ and scCO₂

Microalgae slurry was weighed and placed in a 160 mL continuous stirred batch reactor (Parr, 160 mL T316SS) (CSTR), where, when necessary, deionized water was added to reduce the wt% of the algae. Pre-soak experiments were conducted by adding the required volume of methanol to the sample and allowing the sample to stir for 1 h prior to extraction with CO₂. Following sample input (and pre-soak where indicated) the reactor was connected to the high-pressure system and heated to the operating temperature. Unless otherwise stated, the vessel was constantly stirred with a magnetic stir bar at 500 rpm. The process flow diagram is displayed in Figure 1. IČO₂ was pressurized to the desired operating pressure by a JASCO model PU-980 intelligent HPLC pump. The pump head was maintained at -5°C to ensure that the CO₂ remained in liquid state, and the flow rate was set to the desired value. The IČO₂ extraction was performed at different flow rates (1.5 – 4.0 mL/min), pressures (7.5 – 17.5 MPa), and algal slurry concentrations (0.1 – 17.05 wt% in water). The scCO₂ extraction was performed at analogous extraction conditions to IČO₂ extraction (flow rate of 3 mL/min, at 15 MPa, algal slurry concentration of 14.3 wt% in water and 35 ºC). A backpressure regulator (BPR) (Jasco model 880-81) was used to maintain the pressure throughout the continuous extraction procedures. CO₂, vented from the BPR, was bubbled through methanol to capture extracted lipids. Following extraction, the solvent was removed from the extract in a rotary evaporator at 50°C, and the residue was weighed. Extract percentages (% extract) is calculated as per Equation 1:

\[
\text{% extraction} = \frac{\text{mass of extract (g)}}{\text{Algae paste added (g)/weight % dry mass}}
\]

Equation 1

As such all results are reported as % extracted of dry microalgae mass. Rate of extraction (ext. rate (% ext./h)) is calculated as per Equation 2:

\[
\text{ext. rate (% ext./h)} = \frac{\text{% extraction}}{\text{Time (h)}}
\]

Equation 2

All experimental tests were performed in duplicate and in some instances of the measurements triplicated.
Solid Phase Extraction (SPE)
After extraction at 15.0 MPa at a flow rate of 3 mL/min of either scCO\textsubscript{2} or lCO\textsubscript{2}, extracts were fractionated using LC-NH\textsubscript{2} (500 mg, Supelco) SPE cartridges. A Supelco preppy vacuum manifold was used to facilitate fractionation, with the cartridges preconditioned using 6 mL of hexane. The lipids, dissolved in hexane (minimum volume: 25 mg), were loaded onto the cartridges by percolation through the conditioned cartridges (vacuum free). Three lipid fractions were removed from the loaded cartridge using three different solvent mixtures (flow rate: ~1 ml/min): Fraction A: less polar lipids (triglycerols and diglycerides), designated here as NL (nonpolar lipids). Fraction B: other lipids such as ceramides, designated as “other” (not considered useful in biodiesel production). Fraction C: free fatty acids, designated FFAs.

Fraction A was removed using 5 mL of 15:85 (v/v) ethyl acetate:hexane; fraction B with 4 mL of 23:1 (v/v) chloroform:methanol; and, lastly fraction C with 3 mL of 92:2 (v/v) ethyl ether:acetic acid. The solvent was removed from the three fractions using a rotary evaporator (Ika, RV-10) at 40 °C and further dried in a vacuum oven at 55 °C for 3 h. The dry fractions of extract were measured gravimetrically. Any mass loss was added to the mass of fraction B as unwanted material. Results for SPE fractions are reported as % of the total extract.

Fatty Acid Methyl Ester (FAME) Preparation and Analysis
Methanolysis of microalgae extracts was carried out following a method reported by Paudel et al. (2015). Extracts of \textit{C. vulgaris} (3 mg) (from both lCO\textsubscript{2} and scCO\textsubscript{2} extractions) was solubilized in 1.5 mL of 6.7 mg/ml of H\textsubscript{2}SO\textsubscript{4} in methanol, and 1 mL THF (in 25 mL round bottomed flask). The solution was refluxed for 3 h at 90 °C, neutralized with excess NaHCO\textsubscript{3} and finally extracted with 10 mL of hexane. The extract was dried using a rotary evaporator and re-solubilized in 1.5 mL of hexane. The FAMEs were analyzed by a GC-FID (Perkin Elmer Clarus 680 gas chromatograph (GC)), using an HP-INNOWax column (30 m, 320 µm i.d., 0.25 µm film of polyethylene glycol). Initially the temperature was held at 100 °C for 5 min, followed by a ramp to 250 °C at a rate of 10°C/min and, finally, held at 250 °C for 20 min. Both the injector and detector temperatures were held at 250 °C for the duration of the analysis. Carrier gas (helium) flow rate was maintained at 2 mL/min. Commercially-obtained FAME standards were used for calibration and to determine retention times (stearic methyl ester (SAME), oleic methyl ester (OAME), palmitic methyl ester (PAME) and linoleic methyl ester (LAME)).

\textbf{FAME profile}
It has been previously noted that scCO\textsubscript{2} has a higher affinity for the extraction of saturated fatty acids over unsaturated fatty acids.\textsuperscript{23} Table 1 shows the fatty acid methyl esters formed from the lCO\textsubscript{2} extractions of the microalgae used in the current study compared to scCO\textsubscript{2}.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
 & [PAME] mg/g (extract) & [SAME] mg/g (extract) & [OAME] mg/g (extract) & [LAME] mg/g (extract) \\
\hline
lCO\textsubscript{2} & 10.8 & 18.5 & 8.5 & 6.8 \\
scCO\textsubscript{2} & 11.4 & 19.2 & 8.9 & 7.3 \\
\hline
\end{tabular}
\caption{Fatty acid methyl ester distribution for the extract from \textit{C. vulgaris} using supercritical and liquid CO\textsubscript{2}.}
\end{table}

Fatty acid methyl ester profiles for lCO\textsubscript{2} and scCO\textsubscript{2} extracts show essentially no variation in the selectivity towards the various types of FFA. Additionally it can be seen that through the use of CO\textsubscript{2} extraction it is possible to obtain FFA’s important for the production of bio-diesel.
Results and Discussion

**ICO₂ Extraction**
Comparing the yields of extractions performed using ICO₂ and scCO₂, where all conditions were constant save temperature, shows that the relatively mild conditions using ICO₂ were more effective. Extraction was performed using ICO₂ or scCO₂ at 15.0 MPa, 3 mL/min CO₂ and 14.3 wt% microalgae at 25 °C (ICO₂) and 35 °C (scCO₂). The yield of extract using ICO₂ under these conditions was found to be 4.39 ± 0.16 wt% of dry mass, while for scCO₂ the total extraction was 2.46 ± 0.04 wt%. However, in comparison to the work by Paudel et al., there appears to be a decrease in the total % extracted from wet microalgae compared to freeze dried microalgae. This could be due to variations in cell lysis, reactor design, or extraction conditions such as pressure, temperature, flow rate, and reactor type. However, the use of wet microalgae has the inherent advantage of reducing the required number of process steps, and associated costs, as the need to dry the algal slurry prior to extraction is eliminated.

**Effect of microalgae concentration**
From literature, it is suggested that lipid extraction from microalgae is more effective at 98 wt% as compared to 15 wt% or lower, as this is considered to be the most effective concentration of microalgae for extractions using traditional solvents. However, dewatering processes have been shown to greatly increase the cost of the total extraction process. To gain a better understanding of the effect of the C. vulgaris concentration on the extraction, microalgae was concentrated to 15 wt% (dry mass of microalgae) in water by centrifugation, and was subsequently dispersed into deionized water to obtain a range of concentrations.

The rate of extraction from C. vulgaris at various concentrations is displayed in Figure 2. In all experiments, the pressure, flow rate, temperature, and stirring were kept constant, and the total alga added to the reactor was kept in a similar range (between 0.1 and 0.2 g dry alga). Contrary to expectations, it is apparent in Figure 2 that increasing the concentration of the microalgae greatly decreases the rate of extraction. This could be attributed to a number of different factors, including the loss of fats to the water during the centrifuge step of the process, or inconsistent mixing. However, all extractions at concentrations greater than 0.1 wt% were centrifuged and then dispersed into deionized water. As such, if the decreased yields were due to loss of lipids during centrifuging, the curve would be expected to feature steps and not a steady decrease of rate of extraction with concentration. There is a loss of around 1.5 wt% (by equivalent dry algae mass) to the supernatant solution as a result of the centrifugation of the microalgae (subset samples of supernatant water (from microalgae following centrifugation) were extracted as a blank sample with the use of ICO₂), which is the same for all extractions, apart from raw microalgae samples (0.1 wt%), shown in Figure 2.

**Figure 2:** Effect of C. vulgaris concentration on extraction at constant time, pressure (15 MPa) and flow rate of CO₂ (1.5 mL/min). A) Rate of extraction vs. microalgae concentration; B) total % extraction vs. concentration. On the other hand, the decrease in the extraction yield with increased concentration of microalgae is most likely to be the result of decreased mixing of the ICO₂ with the microalgae suspension. At high algae concentrations, the sample has the consistency of a paste, and therefore the mixing of the algae with ICO₂ is very poor.

**Effect of Mixing**
Figure 3 indicates that the rate of extraction from algae is highly responsive to stirring, where the rate increases...
from about 0.75 %/h to greater than 1.75 %/h with stirring. The effect of mixing on the rate of extraction explains the unexpected results shown in Figure 2. At higher mixing rates, there is better mass transfer from the algal solution to the \( \text{ICO}_2 \), due to improved integration of the two phases. These results indicate that any process design must take into account the mixing within the system for optimal extraction.

**Figure 3:** Rate of *C. vulgaris* extraction under stirred and unstirred conditions at constant time, pressure, algae concentration and flow rate of \( \text{ICO}_2 \) (15.0 MPa, 1.5 mL/min, 25 °C and 0.36 wt%).

**CO\(_2\)** methanol mixture extractions
Both \( \text{ICO}_2 \) and sc\( \text{CO}_2 \) have been shown to selectively extract lipids from microalgae. The yields are lower than those obtained using traditional solvents, and these processes require higher extraction pressures. As a result of this limitation, exploration of other more effective extraction techniques is important. It has previously been shown for freeze-dried algae that \( \text{CO}_2\)-expanded methanol is an effective solvent for lipid extraction. However, its use in the extraction of bio-oils from wet algae is not well studied or understood. Expanded methanol has been shown to have better yields at lower pressure than \( \text{ICO}_2 \) or sc\( \text{CO}_2 \). Compared to traditional organic solvents, CXL methanol has the advantage of reduced temperature and requires smaller volumes of organic solvent. Because of these benefits, the extraction of bio-oils from *Chlorella vulgaris* with \( \text{CO}_2 \) methanol mixtures was conducted at various conditions.

**Effect of methanol volume added to reactor**
When implementing CXL systems as lipid extraction solvents there are several factors to consider. The time required at high pressure and the volume of organic solvent used will impact the energetic and environmental costs of the process. In Figure 4, the effect of the methanol volume added to the microalgae is shown. As the volume of methanol increases, the total yield of bio-oils increases, but this increase diminishes above 100 mL of methanol. The diminishing returns above 100 mL may be due to the concentration of the algae extract in the methanol during extraction and the amount of methanol remaining in the reactor following the extraction. However, solvation factors such as the final polarity of the mixed solvent and the total concentration of available bio-components/bio-oils may be contributing factors.

**Figure 4:** Effect of methanol volume added to *Chlorella vulgaris* at the start of the presoaking step on the total % extracted (mass of extract per mass of dry algae flow rate = 1 mL of \( \text{CO}_2 \) per min, 7.5 MPa).
Selectivity of CO$_2$ extraction

An ideal extraction of FFAs for the conversion into biofuel will have an extract rich in low-polarity lipids such as triglycerides and FFAs. A method using ICO$_2$ for extraction will likely selectively extract such low-polarity. Coincidentally, the extraction of lipids using methods that do not involve ICO$_2$ has a lesser selectivity for non-polar lipids, as seen in Figure 5.

![Figure 5: Percent yields (using subset samples of extract) of lipid fractions for four different extraction methods including MeOH (microalgae soaked in 60 ml MeOH for 24 h), MeOH/CO$_2$ (presoaked in 60 ml MeOH for one hour, 15 MPa extraction for 2 h), CO$_2$ (extraction with ICO$_2$) (15 MPa presoak in the absence of MeOH, 15 MPa extraction for 2 h), and Soxhlet (Soxhlet extraction using a 50:50 (v/v) mix of CHCl$_3$:MeOH for 24 h) analyzed for lipid selectivity of each method using solvents A (15:85 (v/v) mix of ethyl acetate and hexane), B (23:1 (v/v) mixture of chloroform and methanol), and C (92:2 (v/v) mixture of ether and acetic acid).

An extract high in lipids that are found within the neutral fraction is expected, as methanol is a polar solvent. Soxhlet extraction, which is known to have a near-maximized yield, appears to have a very non-specific extracted lipid profile. CO$_2$ alone, and CXL methanol seem to have very similar yields, with the exception of the neutral fraction. The CXL methanol extraction resulted in a higher neutral fraction than pure CO$_2$. This result can be explained by the presence of the methanol, as it shows to have a higher selectivity for neutral lipids. The presence of water in the system greatly increased the ability to extract the free fatty acids from the microalgae when compared to dry algae.

Conclusions

The extraction of lipids from microalgae using ICO$_2$ or CXL methanol has been proposed as an alternative to extraction using traditional solvents. Using ICO$_2$, it is possible to extract lipids from wet microalgae without contaminating the aqueous waste stream with any organic solvent. At higher concentrations of microalgae, the rate of extraction of lipids decreased due to poor mixing. Therefore, any process design needs to take into account the mixing of the CO$_2$ and aqueous layers, as it is a major factor in determining the efficiency of extraction from wet microalgae. The use of both gas expanded solvents and solvent mixtures for the extraction of microalgae has been used previously with varying success. For the use of mixed solvent systems, based on methanol and CO$_2$, it is possible to extract lipids from wet microalgae, with extraction equal to or greater than what has been shown for freeze dried microalgae. Further, this extraction method, with a methanol pre-soaking step, gives greatly increased yields compared to ICO$_2$ and scCO$_2$. Water in the system increased the extraction of free fatty acids, resulting in increased selectivity of ICO$_2$ for FFA’s as compared to scCO$_2$ and reduced selectivity to neutral lipids.
References

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