

Which pre-treatment process yields a higher lipid extraction from *Chlorella vulgaris* prior to Supercritical Carbon Dioxide extraction: the use of microwaves or osmotic-shock based extraction?

Analysis and evaluation of ideas, information and arguments in the selected area of investigation:

Fossil fuels are still the primary source of energy and essential resources. The rapid increase in global warming and immediate demand for energy has catalysed the search for an alternate means of energy production, specifically, renewable energy resources. Among the vast range of renewable energy resources, biodiesel is a promising alternative to fossil fuels since it can be used directly in diesel engines without any effects on the performance and it can also be produced from numerous natural crops. Among the vast selection of crops, microalgae are certainly one of the most preferred, due to its high lipid content per dry weight, swift growth rate and minimalistic land area requirement ([He, 2016](#); [Mata et al. 2009](#); [Ramkumar & Kirubakaran, 2016](#)). While this is the case, growing microalgae for biodiesel can be ethically problematic since land is a finite source, leading to a question of whether land should be used to cultivate microalgae or grow food. However, microalgae can be potentially cultivated in saline water or wastewater, although more research must be conducted to test the effects of the growth environment on the lipid content of microalgae.

Conventionally, lipids are extracted through the use of organic solvents. Common extraction methods include Floch method, Bligh & Dyer method and the Soxhlet technique ([Kumar et al., 2015](#); [Uduman et al., 2010](#)). However, these methods pose a dangerous threat to the environment and humans due to the use of hazardous solvents and high energy consumption ([Taher et al., 2014](#)). Recently, researchers have found a novel approach to extract lipids from microalgae through the use of supercritical carbon dioxide (SC-CO₂). Unlike the previously mentioned methods, SC-CO₂ is an inert, nontoxic and an inexpensive fluid hence proving to be a better alternative. To boost the penetration of solid matrices, several pre-treatments methods can be employed. Thermal pretreatment subjects microalgal cells to microwaves for rapid heating which increases the internal temperature. Therefore, the pressure gradient acting on the cell wall enhances mass transfer rates without lipid degradation ([Uquiche et al., 2008](#)). Alternatively, 'osmotic shock' is also equally advantageous where the abrupt exposure to lower osmotic pressure than in the cells (1M salt solutions), forces water to enter the cell structure. This increases the internal cell pressure causing cell lysis (disintegration of a cell by the rupture of the cell wall or membrane) (Stanbury et al., 2017). While multiple studies have been conducted to test the effects of both pre-treatment methods, none have compared the effects of pre-treatment methods in the process of SC-CO₂ and on *Chlorella vulgaris* microalgal culture. Therefore, through this research, two major questions will be clarified:

- Which pre-treatment method yields the highest percentage of lipids?
- Can supercritical CO₂ extraction replace conventional organic solvent extraction?

Methods of collecting, organising and analysing evidence and/or data:

The method used to conduct this research is a quantitative scientific experiment. A literature review will also be required for a complete analysis of the efficiency and effectiveness of supercritical carbon dioxide as supposed to the conventional solvent-free method. This is because the organic solvent method cannot be executed within school grounds due to health and safety guidelines prohibiting the use of Chloroform and other required solvents. Part of the scientific experiment will be conducted in the school laboratories under the supervision of laboratory technicians. Once the lipids have been extracted, they will be transferred to Monash onsite campus where they will be further analysed qualitatively and quantitatively.

Three distinct sets of experiments will be carried out (with a minimum of three trials of each experiment to increase the reliability and precision of results):

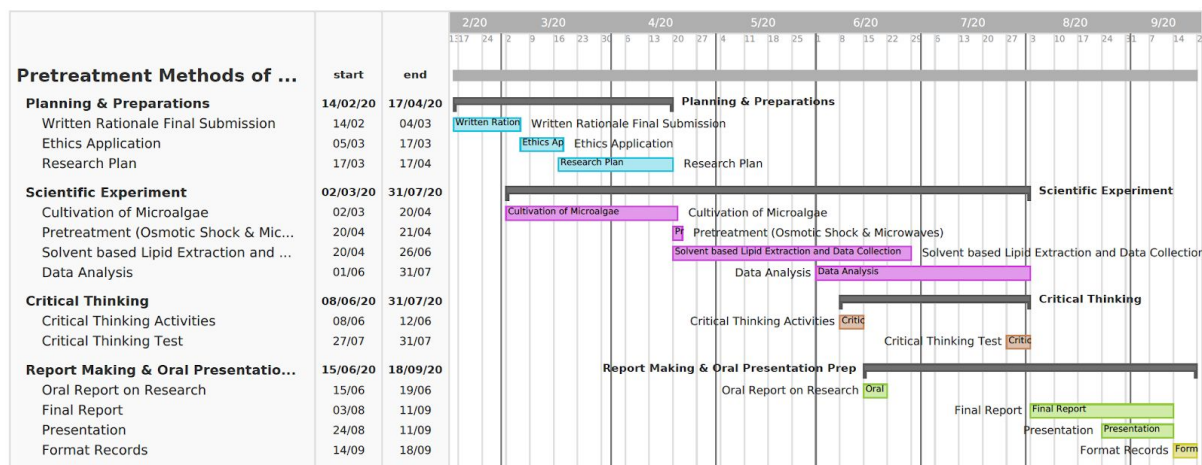
- Thermal (microwave) pretreatment before SC-CO₂ extraction
- Osmotic Shock pretreatment before SC-CO₂ extraction
- SC-CO₂ extraction (controlled) without any pretreatment

However, as mentioned earlier, it is to be noted that another experiment (organic solvent-based extraction) should also have been done, although, as discussed earlier, restrictions at school forbid these types of experiments. Hence, due to this limitation, a literature review will be completed to compensate for this experiment.

Analysis:

The extracts will be analysed using gas chromatography since it offers high sensitivity and mainly because it can provide qualitative and quantitative insight into the composition of the lipid extract. The required apparatus for gas chromatography was provided by the Monash faculty. Helium was used as the primary carrier gas at a flow rate of 36 cm/s, split ratio 1:20, injector and detector temperature was set to 250 °C. The oven temperature was programmed to 50 °C during the first minute, then was increased to 150 °C at a rate 10 °C/min, and from 150 °C to 230 °C at a rate of 3 °C/min, 5 min isothermal, then from 230 °C to 240 °C at a rate of 20 °C/min and finally 10 minutes isothermal. Mass spectra were then acquired in m/z range 50-360 ([Dejoye et al., 2011](#)). The contents of the lipid extract were then identified by comparing their mass spectra and were also quantified by comparing the peak areas of known quantities. This information can then be simplified for non-specialized public and be represented using a table or a pie chart to display the different constituents in the lipid extract and the quantity in which they are present.

Application of research project management, knowledge & skills:



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A review of progress and authentication to the research can be established through journal entries completed every week. This experimental design demands the use of highly specialised equipment to successfully extract and analyse lipids. This includes a supercritical carbon dioxide extractor and a gas chromatograph, which will be used to measure and detect the presence of unique compounds. These pieces of equipment will kindly be provided by Monash University. This research is highly feasible, given that the required equipment can be arranged for.

It is worth to be noted that, due to unforeseen circumstances caused by COVID-19, experiment dates can be delayed. Therefore, if the time allowed for data collection was reduced, the number of trials must be decreased to two or three to save time. However, this would then act as a limitation to this study, nonetheless, the acquired data must be interpreted with caution.

Appendix

Microalgae Cultivation and Harvest

Chlorella vulgaris (var. *viridis* Chodat 1913) was cultivated in John Monash Science School laboratories, by school staff. *C. vulgaris* was situated in standard laboratory conditions (25 °C at 100 kPa).

Through filtration and centrifugation, the biomass was frozen at -70 °C (to avoid contamination and to preserve the integration of its cells) and was successively freeze-dried. As the first step to freeze-drying, the microalgal biomass must be frozen, and must later follow sublimation of ice. This thereby dries the microalgae under a vacuum (25 Pa) and at -80 °C. The ice is then vaporized and the biomass is then recovered. Upon the sublimation of all of the ice, the microalgal paste is finally dried, where it is left to rest at room temperature for two to six hours (to reduce the residual moisture).

Pretreatment

Microwave Pretreatment:

This procedure was performed using a classical Mwave-5000 microwave oven (note: model is subject to change due to availability). The power output of the microwave was variable up to 1000W with a frequency of 2450 MHz. To analyse the effects of microwave pretreatment at a greater depth, three different power outputs were selected and applied: 600 W, 800 W and 1000W. Upon completion, these can be compared against the increase in the percentage of lipids extracted using a bar chart to reveal the effects of various power outputs. 100 ± 0.05g of pre-dried microalgae was weighed using a balance and was set in a Pyrex bowl. It was then placed into the microwave oven and was treated over six cycles: 1 min irradiation stage and was then allowed to be cooled to 25 °C, to ensure that the cells did not completely disintegrate ([Dejoye et al., 2011](#)).

Osmotic Shock Pretreatment:

C. vulgaris was also alternatively disrupted using osmotic shock method. Three vials were prepared, each with 6 ml of 5%, 10% and 15% sodium chloride solution, and were respectively labelled. 100 mg ± 0.05g of microalgal biomass was then suspended into each vial and was vortexed for 4 mins, and subsequently incubated for 48 hours at SLC.

Osmotic shock pressure obeys a relationship of the same form of the ideal gas equation and was calculated according to the Morse equation:

$$(P_i) = iMRT$$

- ❖ (Pi) = cell disrupting pressure (atm or kPa)
- ❖ i = dimensionless van't Hoff factor
- ❖ M = Molar concentration of sodium chloride (mol/L)
- ❖ R = Universal gas constant (0.0821 L*atm/K/mol)
- ❖ T = absolute temperature (K)

The cell disrupting power was calculated using an online osmotic pressure calculator, designed by Georgia State University ([Nave, n.d.](#)).

Lipid Extraction

Supercritical CO₂ Extraction:

Supercritical carbon dioxide experiments were performed on HA221-40-100 apparatus (lent by Monash University) featuring a thermostatic extractor and separators connected in series (note: model is subject to change due to availability). Each operation of SC-CO₂ involved the introduction of 100 ± 0.05 g of dried *C. vulgaris* into the filter basket within the supercritical reactor. These tasks were conducted at isothermal and isobar conditions at 40-70 °C and 20-28 MPa. The carbon dioxide flow rate was set to 10 kg/h. Each extraction was performed multiple times and was nine hours in length. Lipid extracts were also collected every 30 mins for the development of a kinetic profile diagram. Lipid extracts obtained were stored in a nitrogen atmosphere lower than -20 °C to preserve the lipid oxidation ([SB, M & DU, 2011](#)).

Bibliography

- Dejoye, C., Vian, M. A., Lumia, G., Bouscarle, C., Charton, F., & Chemat, F. (2011). Combined extraction processes of lipid from chlorella vulgaris microalgae: Microwave prior to supercritical carbon dioxide extraction. *International Journal of Molecular Sciences*, 12(12), 9332-9341.
doi:<http://dx.doi.org.ezproxy.lib.monash.edu.au/10.3390/ijms12129332>
- He, B. (2016). Advances in emission characteristics of diesel engines using different biodiesel fuels. Retrieved 1 May 2020, from
<https://www.sciencedirect-com.ezproxy.lib.monash.edu.au/science/article/pii/S1364032116001234?via%3Dihub>
- Kumar, R., Rao, P. and Arumugam, M. (2015). *Lipid extraction methods from microalgae: a comprehensive review*. [online] Frontiers in Energy Research. Available at:
<https://www.frontiersin.org/articles/10.3389/fenrg.2014.00061/full>.
- Mata, T., Martins, A., & Caetano, N. (2009). Microalgae for biodiesel production and other applications: A review. Retrieved 1 May 2020, from
<https://www.sciencedirect-com.ezproxy.lib.monash.edu.au/science/article/pii/S1364032109001646?via%3Dihub>
- Nave, R. Osmotic Pressure Calculation. Retrieved 30 May 2020, from
<http://hyperphysics.phy-astr.gsu.edu/hbase/Kinetic/ospcal.html>
- Ramkumar, S., & Kirubakaran, V. (2016). Biodiesel from vegetable oil as alternate fuel for C.I engine and feasibility study of thermal cracking: A critical review. Retrieved 1 May 2020, from
<https://www.sciencedirect-com.ezproxy.lib.monash.edu.au/science/article/pii/S0196890416302096>
- SB, G., M, R., & DU, S. (2011). The combined extraction of sage (*Salvia officinalis* L.): ultrasound followed by supercritical CO₂ extraction. Retrieved 1 May 2020, from <https://www.ncbi.nlm.nih.gov/pubmed/20634117>
- Stanbury, P., Whitaker, A., & Hall, S. (2017). Chapter 10 - The recovery and purification of fermentation products. Retrieved 30 May 2020, from
<https://www.sciencedirect.com/science/article/pii/B9780080999531000107>

- Taher, H., Al-Zuhair, S., Al-Marzouqi, A., Haik, Y., Farid, M., & Tariq, S. (2014). Supercritical carbon dioxide extraction of microalgae lipid: Process optimization and laboratory scale-up. Retrieved 1 May 2020, from <https://www-sciencedirect-com.ezproxy.lib.monash.edu.au/science/article/pii/S0896844613003963?via%3Dihub>
- Uduman, N., Qi, Y., Danquah, M., Forde, G., & Hoadley, A. (2010). Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. Retrieved 1 May 2020, from https://aip.scitation.org/doi/full/10.1063/1.3294480?casa_token=_SvXhv3c480AAAA%3A2CU9EIVqX8adGtiwCktNnHzWI1NDiZW8fl4I28NVcOdCVF9kqAc_hDTLa3dXYqFrSI_Oonf62cN82oQ
- Uquiche, E., Jeréz, M., & Ortíz, J. (2008). Effect of pretreatment with microwaves on mechanical extraction yield and quality of vegetable oil from Chilean hazelnuts (*Gevuina avellana* Mol). Retrieved 28 April 2020, from <https://www-sciencedirect-com.ezproxy.lib.monash.edu.au/science/article/pii/S1466856408000519>