

2020-07-01

## Enzymatic in-situ transesterification of neutral lipids from simulated wastewater cultured *Chlorella emersonii* and *Pseudokirchneriella subcapitata* to sustainably produce fatty acid methyl esters

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### Recommended Citation

Priyanka, P., Kinsella, G. K., Henehan, G. T., & Ryan, B. J. (2020). Enzymatic in-situ transesterification of neutral lipids from simulated wastewater cultured *Chlorella emersonii* and *Pseudokirchneriella subcapitata* to sustainably produce fatty acid methyl esters. *Bioresource Technology Reports*, 100489.

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Enzymatic *in-situ* transesterification of neutral lipids from simulated wastewater cultured *Chlorella emersonii* and *Pseudokirchneriella subcapitata* to sustainably produce Fatty Acid Methyl Esters.

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## Highlights

- Simulated wastewater generated biomass and neutral lipids in microalgae.
- Microalgae metabolic engineering enhanced neutral lipid production.
- Lipases from two novel *Pseudomonas* strains used for *in-situ* transesterification.
- Gas chromatography determined the fatty acid methyl esters (FAMES) for composition.

## Abstract

Alternative, more sustainable and environmentally positive, sources of energy are one of the current global challenges. One approach to achieving more sustainable sources of energy is to use waste from one system as a raw material for energy production, following the circular

biosystem philosophy. This study successfully adopted this approach whereby microalgae strains *Chlorella emersonii* and *Pseudokirchneriella subcapitata* were bioprospected and metabolically engineered in simulated wastewater supplemented with glucose to produce neutral lipids. Using a two-step cultivation approach neutral lipid content was enhanced in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* biomass. Via *in-situ* transesterification, these neutral lipids were subsequently bioconverted to biodiesel feedstock fatty acid methyl esters using novel solvent stable lipase(s) from *Pseudomonas reinekei* and *Pseudomonas brenneri*. The culturing of appropriate microalgae on wastewater, and bioconversion via organo-stable lipases may provide a commercially viable and sustainable biodiesel feedstock to help address the current global energy challenge.

#### Keywords

Lipase, *In-situ* transesterification, microalgae, fatty acid methyl esters, simulated waste-water

#### 1. Introduction

Fossil fuels, such as petrol, coal, and natural gas, contribute 88% of global energy consumption; with the transport sector subsequently being a major contributor of CO<sub>2</sub> global emissions, which, by 2050 is estimated to increase to 2 billion vehicles (Balat and Balat, 2010). More urgently, by 2030, the emission of total anthropogenic greenhouse gases is projected to increase by 23 percent (Ullah *et al.*, 2015). This environmental concern, coupled with the growing global energy demand, has resulted in increased interest in the production of environmentally friendly and sustainable liquid fuels, such as biofuels, as a suitable alternative source of energy (Milano *et al.*, 2016). Biofuels, comprising Fatty Acid Methyl Esters (FAMES), are typically produced from

renewable biological oils and fats, such as vegetable oil, animal fats or nonedible plant feed stocks and can sometimes be used directly in existing engines with little or no modification (Scott *et al.*, 2010). Currently, alternative sources of lipids, such as microalgae, are currently being bioprospected.

Microalgae; due to a short harvesting cycle and high growth rates allow rapid accumulation of significant amounts of lipids, with appropriate culturing conditions. Microalgae are highly adaptable towards their surrounding environment; they do not require fertile land and can therefore be grown almost anywhere, even on treated sewage or saltwater. Since microalgae require CO<sub>2</sub> to grow and can efficiently remove phosphates and nitrates from wastewater, they are an appropriate biomass for bioremediation and biofixation (Lam and Lee, 2012). The lipid content in microalgae varies from 20-40% biomass dry weight, however, lipid content as high as 85% biomass dry weight has also been reported in certain microalgal strains (Mairet *et al.*, 2011). Optimizing the metabolic pathways of microalgae cells can increase the lipid content (Lowrey, Brooks and McGinn, 2015). Therefore, utilising a short harvesting cycle, coupled to metabolic pathway optimisation to produce higher lipid content and with continuous harvesting suggests that microalgae may be very good candidates for sustainable fuel production (Miao and Wu, 2006). In the current study this hypothesis has been explored where two microalgae strains, *Chlorella emersonii* and *Pseudokirchneriella subcapitata*, were cultured and metabolically engineered in simulated wastewater for neutral lipids.

Nile Red dye was used for quantification of neutral lipids in the current study as a high correlation between neutral lipid content (as per the gravimetric method) and Nile Red fluorescence has been established and the assay is used as the quantitative analysis method for neutral lipid quantification in a variety of microalgae, such as *Chlorella* sp. (Chen *et al.*, 2009;

Huang, Chen and Chen, 2009). Fluorescence and staining kinetics, in addition to microalgae species also dependent on the composition and structure of the microalgal cell wall. Robust and thick cell walls (particularly in green algae and nutrient starved microalgae) act as a barrier; preventing efficient penetration of Nile Red in cells and neutral lipid staining (Doan and Obbard, 2011; Guzman *et al.*, 2011; Pick and Rachutin-Zalagin, 2012). Hence, for the estimation of neutral lipids in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* in the present study, a Nile Red assay was developed.

Microalgae lipid extraction usually follows two steps: cell disruption and solvent extraction. Disruption of the microalgal cell wall for the extraction of oil in an extraction solvent increases the energy requirement and cost due to the additional steps of dewatering and drying of microalgal cells (Ronald, Micheal and Paul, 2012). Several mechanical, chemical or combinational cell (ultrasound, high-pressure homogenization, bead-beating, osmotic shocks) methods have been suggested to facilitate oil extraction from algal cells (Halim *et al.*, 2012; Ronald, Micheal and Paul, 2012). However, due to increased processing costs and time, other transesterification methods are required. In the current study, FAMES, as a precursor for biodiesel, were synthesized via *in-situ* transesterification using novel lipases and the neutral lipids from the biomass of *Chlorella emersonii* and *Pseudokirchneriella subcapitata*.

Lipases can catalyse both hydrolysis and synthesis of long-chain acylglycerols and most importantly can catalyse biodiesel production since they are the only enzymes that catalyse the synthesis of esters (i.e. transesterification (Jaeger and Reetz, 1998). Traditionally, the majority of industrial scale transesterification reactions are carried out in organic solvents due to the ease of solubility of the non-polar lipid substrates. However, most lipases are denatured in organic solvents and, therefore, lose their catalytic activity. In an alternative approach, this study

employed novel, solvent stable lipases from *P. reinekei* (H1) and *P. brenneri* (H3) for the *in-situ* transesterification of neutral lipids from *Chlorella emersonii* and *Pseudokirchneriella subcapitata* to produce sustainable, economical and environmentally positive FAMEs that were chemically comparable to commercially available biodiesel FAMEs mix.

## 2. Materials and methods:

### 2.1. Chemicals and materials:

*Chlorella emersonii* (CCAP 211/8C) was supplied by the Culture Collection of Algae and Protozoa (CCAP) while *Pseudokirchneriella subcapitata* was supplied by RESC ExoTox lab of the FOCAS Research Institute, TU Dublin. The GC column for FAMEs analysis was purchased from Bruka. All other chemicals were of analytical grade and were purchased from Sigma-Aldrich. Lipases for enzymatic *in-situ* transesterification were isolated from *P. reinekei* (Priyanka *et al.*, 2019) and *P. brenneri* (H3; Priyanka, 2020).

### 2.2. Neutral lipid synthesis from Microalgae

#### 2.2.1. Microalgae Culturing conditions

Bold basal medium, with 3-fold nitrogen and vitamins (3N-BBM+V modified), was prepared as per manufacturer's instructions. Immediately upon delivery; *Chlorella emersonii* and *Pseudokirchneriella subcapitata* were transferred to 3N-BBM+V media in 1:10 ratio (culture: media). Both cultures were grown in a shaker incubator (18°C, with a 16h:8h light: dark cycle and a constant mixing at 120rpm). After 10 days, fresh 3-BBM+V media was added and both strains were grown until significant growth was observed for storage and experimentation. The microalgal culture was maintained by routine serial transfer.

Synthetic secondary wastewater, as per EU composition (DIN 38412-26), was prepared (Table 1) and autoclaved. Stock solutions were maintained separately and under sterile condition were mixed prior to use. Simulated wastewater without nitrogen components (i.e. without Stock 1), was prepared by combining Stock 2 and Stock 3 in a sterile autoclaved conical flask. The final volume of the culture media was made up by the addition of autoclaved distilled water.

### 2.2.2. *Biomass generation and neutral lipid production:*

Microalgae cultures were grown in simulated wastewater media under three different conditions. Growth in autotrophic condition involved photosynthesis (presence of light and CO<sub>2</sub>); in mixotrophic condition involved photosynthesis along with 1% (w/v) glucose as carbon source; while heterotrophic condition eliminated photosynthesis (cultured in the dark) but utilised only 1% (w/v) glucose as a carbon source. The culturing conditions resulting in highest biomass were subsequently used for the generation of microalgal biomass as the first step of neutral lipids production. The microalgal biomass generated in the relevant growth media was harvested by centrifugation at 5,000\*g for 5 mins at 18°C and was washed twice with sterile autoclaved distilled water before further experimentation. The washed cell pellet was transferred to simulated nitrogen deficient wastewater with and without 1% (w/v) glucose. The culture was mixed thoroughly by shaking the conical flask in a rotatory motion for uniform cell distribution. Neutral lipid production was then followed at 18°C, 16h:8h (light: dark), 120 rpm.

### 2.2.3. *Nile Red assay:*

The Nile Red assay, for the estimation of neutral lipids in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* was carried out using fixed number of cells (0.6 OD<sub>@590nm</sub>; with

1 O.D.<sub>@590nm</sub>=10<sup>7</sup> cells/ml) from the simulated wastewater growth media. The cells were transferred to nitrogen deficient wastewater media and after one week of culturing (18°C with 16h:8h (light: dark) cycle, 120rpm) in nitrogen deficient wastewater; 10mls of culture were aseptically removed into a universal tube. The culture was centrifuged at 5,000\*g for 5mins and the culture media was discarded. The microalgal cell pellet was then mixed thoroughly in 10mls of double distilled water and, subsequently, 50µl of this was used for assay. The assay was carried out in quintuplets utilising a flat bottom transparent 96-well microtiter plate. Into each well 100µl of double distilled water, initially, and 50µl of microalgae culture, subsequently, were added followed by 100µl of Nile Red dye. Neutral lipid estimation in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* was carried out using Nile Red dye with 5µg/ml and 10µg/ml concentrations respectively in 20% (v/v) DMSO.

For quantification of neutral lipids, a working range of triolein standard (between 1µg/ml to 15µg/ml) was prepared in neat chloroform. For the quantification, in individual well of a 96-well plate, 100µl of ddH<sub>2</sub>O, 100µl of 10µg/ml Nile Red (in 20% v/v DMSO) and 50µl of the respective triolein concentration was mixed, in quintuplet. Fluorescence intensity was measured at 530nm excitation and 580nm emission wavelength after 5mins of incubation. The fluorescence intensity from various concentrations of triolein was used for standard curve preparation and for neutral lipids quantification in microalgae biomass.

### 2.3. *In-Situ Transesterification*

*In-situ* transesterification of microalgae biomass was executed as per Tran and co-workers' protocol (Tran *et al.*, 2012) with minor deviations. In brief, 0.5g of freeze-dried microalgae biomass was mixed with 20ml of neat methanol and sonicated at 70 amplitude for 20mins, using

QSonica Q55 sonicator. After sonication, methanol was evaporated by placing the container in a fume hood for 60mins to obtain an oil-containing slurry. This slurry was later mixed, by vortexing for 5mins with 5ml of *n*-hexane. 500IU of lipase/g of microalgae oil was subsequently added to the mixture along with 5ml of methanol to initiate the transesterification reaction at 40°C for 72h. To overcome the issue of solvent evaporation; each reaction was carried out in a sealed glass container. After transesterification, the sample was centrifuged at 5,000\*g for 10mins and the upper solvent layer containing FAMES was pipetted carefully to a clean sealed glass container. The FAMES generated were analysed by Thin Layer Chromatography (TLC) and Gas Chromatography (GC). The yield of biodiesel from microalgae biomass was calculated as per (Cao *et al.*, 2013);

$$\text{Biodiesel Yield (\%)} = \frac{\text{Biodiesel Mass (g)}}{\text{Algae mass (g)} \times \text{Oil content (\%)}} \times 100$$

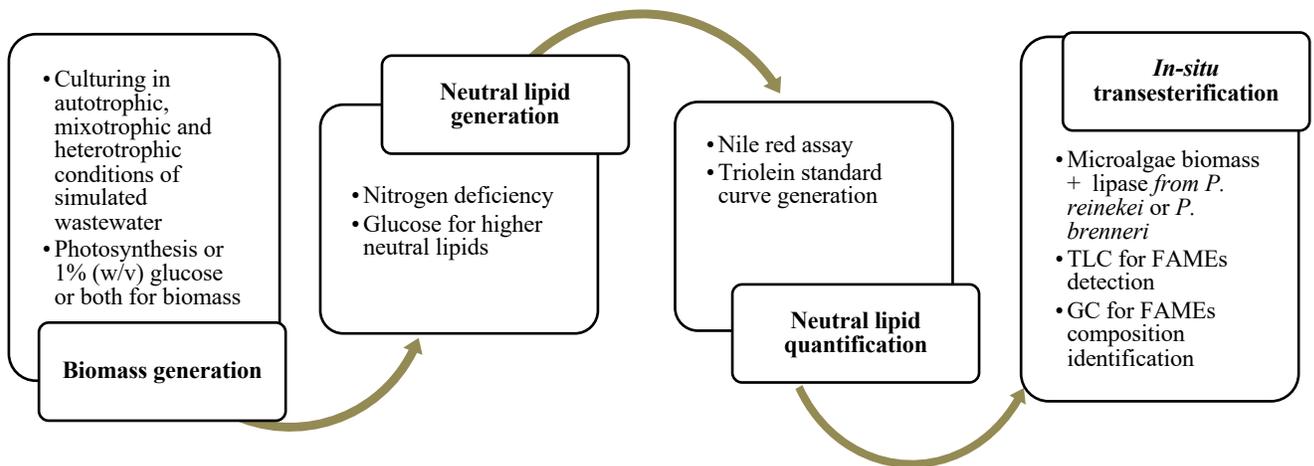
#### 2.4. Thin Layer Chromatography and Gas Chromatography

Post transesterification TLC FAMES detection was carried out as per Kim and colleagues (Kim *et al.*, 2014) with no deviations. In brief, a 90:10 (v/v) *n*-hexane: diethyl ether solvent mix was used as the mobile phase and after full development of TLC plate, the FAME spots were visualized using a 10% (v/v) ethanoic phosphomolybdic acid spray, followed by drying at 105°C for 5mins.

The GC method of (David, Sandra and Vickers, 2005) was used for FAME analysis. In brief, a Bruka GC column (0.25mm Internal Diameter and 30m Length) operating as part of a 0.25µm particle size Scion-436GC machine with FID detector, was used for FAME analysis. FAMES

identification was carried out by comparing their Retention Time (RT) of the product with RT of a 37-component FAME standard mix (Sigma).

## 2.5. Experimental Scheme



## 3. Results and Discussion:

### 3.1. *Chlorella emersonii* and *Pseudokirchneriella subcapitata* biomass generation

Mixotrophic cultivation improves the efficient use of light or eliminates its requirement by cells; CO<sub>2</sub> and organic carbon are used in photosynthetic and respiratory metabolism simultaneously, resulting in a synergistic effect of autotrophic (carbon dioxide fixation by Calvin-Bensen cycle) and heterotrophic processes (Yeesang and Cheirsilp, 2014). Though various sources of organic carbon (glucose, acetate, sucrose etc.) have been used for mixotrophic and heterotrophic growth conditions, the most prevalent source is glucose due to its availability (Perez-Garcia *et al.*, 2011).

Furthermore, glucose is one of the final photosynthesis products; and would suggest that any photosynthetic microorganism must be able to incorporate it in its metabolism (Yeesang and Cheirsilp, 2014). Therefore, in the current study glucose was used as a source of organic carbon for mixotrophic and heterotrophic growth conditions for the microalgae strains. Figures 1 and 2 depict the growth curves, in different wastewater culturing conditions, for *Chlorella emersonii* and *Pseudokirchneriella subcapitata* respectively.

Mixotrophic growth conditions of microalgae have shown promising results for increasing both biomass and lipid content in many microalgae (Lee *et al.*, 1996; Yamane *et al.*, 2001; Lowrey, Brooks and McGinn, 2015; Ratnapuram, Vutukuru and Yadavalli, 2018). A maximum biomass concentration of 11.1g/L was observed in mixotrophic conditions, utilising glucose as organic carbon, for *Chlorella sorokiniana* compared to a 2.2g/L yield in photoautotrophic conditions for the same species (Lee *et al.*, 1996). Likewise, maximum biomass of 2.46g/L was also obtained for *Botryococcus braunii* cultured in mixotrophic conditions, where 5g/L of glucose was used as an organic carbon source (Yeesang and Cheirsilp, 2014). In the current study, to obtain a higher biomass of *Chlorella emersonii* and *Pseudokirchneriella subcapitata*, a 5-day and 8-day mixotrophic culturing mode respectively was found to be optimum. Furthermore, a difference in physiological appearance was observed for both the cultures for different growth conditions (see **Appendices 1** and **2**), indicating a reduced production of chlorophyll in mixotrophic and heterotrophic mode.

### *3.2. Neutral lipid production in nitrogen deficient conditions*

The ability of microalgae to survive or proliferate over a wide range of environmental conditions can be due to unusual cellular lipids, as well as an ability of microalgae to modify lipid

metabolism, in response to altered environmental conditions (Thompson, 1996; Wada and Murata, 1998; Gouveia and Oliveira, 2009). Shen and colleagues (Shen *et al.*, 2009) have demonstrated that limiting certain nutrients (e.g. nitrogen) can result in higher lipid production and storage, as the microalgal cells respond to the stress conditions imposed. Spoehr and Milner (Spoehr and Milner, 1949), first demonstrated that lipid content could be increased (from 5% to 85%) in nitrogen starved *Chlorella pyrenoidosa* culture, and since then nutrient deficiency/depletion (particularly nitrogen) has been regarded as the most efficient approach to increase lipid content in algae (Rodolfi *et al.*, 2009).

In the current study, the depletion of nitrogen sources from simulated wastewater media in cultivation of *Chlorella emersonii* (Figure 3) and *Pseudokirchneriella subcapitata* (Figure 4) resulted in the production of maximum neutral lipids after 6 and 12 days of incubation respectively. The increase in lipid production under nitrogen limited conditions is typically due to the disorganization of lipid synthesizing enzymes being less than compared to carbohydrate synthesizing enzymes; thus, the major proportion of carbon is fixed in lipids (Becker, 1994). A high concentration of the appropriate nutrients is necessary for a high growth rate and maximum biomass; but accumulation of a large concentration of lipids normally cannot take place during such growth. Culturing under a nitrogen deficient conditions leads to higher lipid accumulation, but with lower biomass, and vice-versa. In order to balance the challenges associated with simultaneous production of biomass and neutral lipids; a two-stage cultivation process can be followed, as executed in this study. The first step of cultivation focuses on microalgal biomass generation by providing optimum nutrients. In the second stage, the biomass from the first stage is harvested, washed with deionized water to remove any traces of nitrogen or other nutrients from stage one and then the biomass is grown in nitrogen deficient media.

The absence of nutrients (nitrogen or phosphorous) in stage two of the cultivation process reduces the biomass however; incorporation of an organic carbon source like glucose or sodium acetate is known to enhance the lipid production along with biomass. A similar two-stage approach was also carried out to enhance neutral lipid content in *Nannochloropsis gaditana*, *Tetraselmis chuii*, *Tetraselmis suecica* and *Phaeodactylum tricornutum* (Pedro *et al.*, 2013) and in *Chlorella vulgaris* (Cui *et al.*, 2017). Heterotrophic lipid generation conditions in *Chlorella protothecoides* have demonstrated an increase in lipid content from 15% to 55% (Miao and Wu, 2006). Likewise, the addition of 4g/L sodium acetate in nitrogen and phosphorous deficient media increased total lipid content by 93% in *Chlamydomonas reinhardtii* (Yang *et al.*, 2018). This mirrors the observations in the current study; following the two-step cultivation for *Chlorella emersonii* and *Pseudokirchneriella subcapitata* an increase in lipid content was observed when 10g/L of glucose was added in a nitrogen starved environment. In autotrophic nitrogen deficient media 6 days and 13 days of cultivation generated maximum neutral lipid content, however; in a mixotrophic condition a 45% and 64% increase in neutral lipid content was achieved after 13 days and 21 days of cultivation for *Chlorella emersonii* (Figure 3) and *Pseudokirchneriella subcapitata* (Figure 4) respectively.

Microscopic examination of microalgal cells indicated *Chlorella emersonii* to be spherical and *Pseudokirchneriella subcapitata* to be sickle shaped (Appendix 3 (a) and 4 (a)). Treatment of microalgal cells with 20% (v/v) DMSO containing Nile Red generated fluorescence when cells were observed under green excitation zone (530nm) and a red emission zone (604nm; Appendix 3 (b), 4 (b)). The presence of fluorescence after excitation of Nile Red treated microalgal cells indicated the presence of neutral lipids in the microalgal strains.

Using the standard curve generated by a five-point calibration of triolein (Appendix 5), the concentration of triacylglycerols in *Chlorella emersonii* and *Pseudokirchneriella subcapitata* after following the two step cultivation; 13 and 21 days of culturing in nitrogen deficient media with 1% (w/v) glucose was calculated to be  $0.61 \pm 0.017$  mg/mg and  $0.31 \pm 0.006$  mg/mg of biomass respectively.

### 3.3. *In-situ* transesterification of microalgae lipids to produce Fatty Acid Methyl Esters

#### *In-situ* transesterification of *Chlorella emersonii* and *Pseudokirchneriella subcapitata*

lyophilised biomass containing triacylglycerides (TAGs) and free lipase from *P. reinekei* (H1) and *P. brenerri* (H3) after 72h of incubation at 40°C resulted in FAMES generation, as detected by TLC (Appendix 6). The transesterification time of 72h in the current study can be reduced by either increasing the ratio of methanol: oil or by increasing the amount of lipase used in the transesterification reaction (Jagadale and Jugulkar, 2012; El-batal *et al.*, 2016; Rangel-Basto *et al.*, 2018; Lv, Sun and Jinming Lu, 2019). Though the generation of FAMES from *Chlorella emersonii* and *Pseudokirchneriella subcapitata* biomass was observed within 24h of incubation, a 72h incubation was carried out to achieve maximum yield.

Alcohol is required for *in-situ* transesterification during the oil extraction step and the transesterification of microalgal neutral lipids; therefore larger quantities of alcohol are used in *in-situ* transesterification reactions compared to the commercial biodiesel production process (Dianursanti, Religia and Wijanarko, 2015; Park, Park and Lee, 2015). For *in-situ* transesterification, the use of co-solvent increases the biodiesel yield. Co-solvents also aid oil extraction from microalgal cells and enhance the contact of microalgal oils with the esterification reagent (Cao *et al.*, 2013). Hexane and *t*-butanol are the most common co-solvents used during *in-situ* enzymatic transesterification reactions (Heitz *et al.*, 2016). As the lipase from *P. reinekei*

(H1) and *P. breunneri* (H3) showed enhanced lipolytic activity in *n*-hexane, *n*-heptane and cyclohexane (Priyanka *et al.*, 2019), any of these solvents could be used as the co-solvent of choice for the *in-situ* transesterification reaction of *Chlorella emersonii* and *Pseudokirchneriella subcapitata* neutral lipids. The use of *n*-hexane with methanol 1:1 (v/v) has been reported to increase FAME conversion by 94% (Dianursanti, Religia and Wijanarko, 2015), and therefore, a 1:1 (v/v) ratio of methanol and *n*-hexane were used for *in-situ* biodiesel/FAME synthesis from *Chlorella emersonii* and *Pseudokirchneriella subcapitata* in this study. The yield of FAMEs generated after *in-situ* transesterification was calculated to be 24%.

After analysis via GC, a higher percentage of C18:0 unsaturated fatty acid methyl esters was observed in the FAMEs generated from the neutral lipids of *Chlorella emesonii* and *Pseudokirchneriella subcapitata*; irrespective of the lipase used for the transesterification reaction (Table 2). This result echoes previous studies where nitrogen deficient culturing induces the accumulation of C16:0 and C18:0 unsaturated fatty acid methyl esters in microalgae strains (Rodolfi *et al.*, 2009; Tang *et al.*, 2011). A different percentage of FAMEs composition was obtained when different lipase sources were used for transesterification of same microalgae biomass. This FAMEs composition variation could be due to different substrate specificities of the lipases used. Increased accumulation of C18 unsaturated fatty acid methyl esters under a nitrogen starvation condition has also been reported in *Nannochloropsis* sp. (Rodolfi *et al.*, 2009), *Chlorella minutissima* UTEX 2341 (Tang *et al.*, 2011) and *Nannochloropsis oculata* (Su *et al.*, 2011). Appendix 7 is a GC chromatogram of FAMEs generated after *in-situ* transesterification of neutral lipids from *Chlorella emersonii* using lipase from *P. reinekei* (H1).

FAMEs generated after the transesterification of *Chlorella emersonii* and *Pseudokirchneriella subcapitata* contain higher percentage of unsaturated fatty acid methyl esters though can suffer

from higher oxidation but can attribute towards a good cloud point (CP) and pour point (PP). The generated FAMES, if blended with appropriate percentages of saturated fatty acid methyl esters, can be used as a vehicle fuel substitute.

#### 4. Conclusion:

This study demonstrated maximum biomass generation from *Chlorella emersonii* and *Pseudokirchneriella subcapitata* was achieved under mixotrophic culturing conditions in simulated wastewater supplemented with 10g/L glucose (1% w/v). Neutral lipid accumulation was subsequently induced through nitrogen depletion and converted, via *in-situ* transesterification, to FAMES which comprised primarily unsaturated fatty acid methyl esters as revealed by GC analysis. Although the final transesterification product generated was pure, further analysis of the analytical properties are required to identify future commercial applications as a biodiesel substitute. This study provides a proof of concept for utilizing natural environmentally friendly feeds for the generation of biofuels.

#### Acknowledgement

This research was funded by *Fiosraigh* Scholarship (PB04146) granted by Technological University Dublin.

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## Figure Captions

Figure 1: Growth curve of *Chlorella emersonii* following three different modes of culturing (Auto; Autotrophic, Mixo; Mixotrophic and Hetro; Heterotrophic). The maximum biomass was produced after 5 days of culturing in mixotrophic mode. Data represented here are the average of three different trials, with the standard deviation noted as error bars.

Figure 2: Growth curve of *Pseudokirchneriella subcapitata* following three different modes of culturing (Auto; Autotrophic, Mixo; Mixotrophic and Hetro; Heterotrophic). The maximum biomass was produced after 8 days of culturing in mixotrophic mode and 10 days of culturing in autotrophic mode. The data represented here are the average of three different trials, with standard deviation noted as error bars.

Figure 3: The effect of nitrogen deficient media with, and without, 1% (w/v) glucose on the production of neutral lipids in *Chlorella emersonii* as determined by the bespoke Nile Red assay. The data represented here are the average of three different experiments, with five assay values, and the error bars noted are the standard deviation.

Figure 4: The effect of nitrogen deficient media with, and without, 1% (w/v) glucose on the production of neutral lipids in *Pseudokirchneriella subcapitata* as determined by the Nile Red assay. The data represented here are the average of three different experiments, with five assay values, and the error bars noted are the standard deviation.

## Tables and Figures

Table 1: Composition of simulated wastewater as per EU (DIN 38412-26).

Stock No.	Stock solution	Conc. (g/L)	Vol (ml) Stock for 1L wastewater	Final conc. (mg/L) In wastewater
1	Peptone	8	20	160
	Meat Extract	5.5		110
	Urea	1.5		30
2	NaCl	0.35	20	7
	CaCl <sub>2</sub> ·2H <sub>2</sub> O	0.2		4
	MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.1		2
3	K <sub>2</sub> HPO <sub>4</sub>	1.4	30	28

Table 2: Summary of Fatty Acid Methyl Esters composition following GC analysis and comparison to FAME standard. Also detailed are the other saturated fatty acid esters (OSFA) and other unsaturated fatty acid esters (OUFA) noted after *in-situ* transesterification of *Pseudokirchneriella subcapitata* and *Chlorella emersonii* neutral lipids by purified lipase from *P. reinekei* (H1) and *P. brenneri* (H3) respectively.

		% Fatty acid Composition							
Neutral lipid source	Lipase source	C17:1	C18:0	C18:1	C18:2	C18:3	C20:0	∑OSFA	∑OUSFA
<i>Pseudokirchneriella</i>	<i>P. reinekei</i>	14.61±1.46	0.73±0.07	2.83±0.28	22.17±2.22	5.39±0.54	5.83±0.58	22.59±2.26	25.86±2.59
<i>subcapitata</i>	<i>P. brenneri</i>	7.001±0.70	0.00	0.104±0.02	0.00	76.93±7.69	0.00	4.1±0.41	11.9±1.19
<i>Chlorella emersonii</i>	<i>P. reinekei</i>	5.403±0.54	0.396±0.04	0.073±0.01	0.00	82.02±8.20	7.099±0.71	0.59±0.07	4.43±0.44
	<i>P. brenneri</i>	6.803±0.68	0.00	0.074±0.01	0.00	56.649±5.66	0.00	10.39±1.04	26.08±2.61

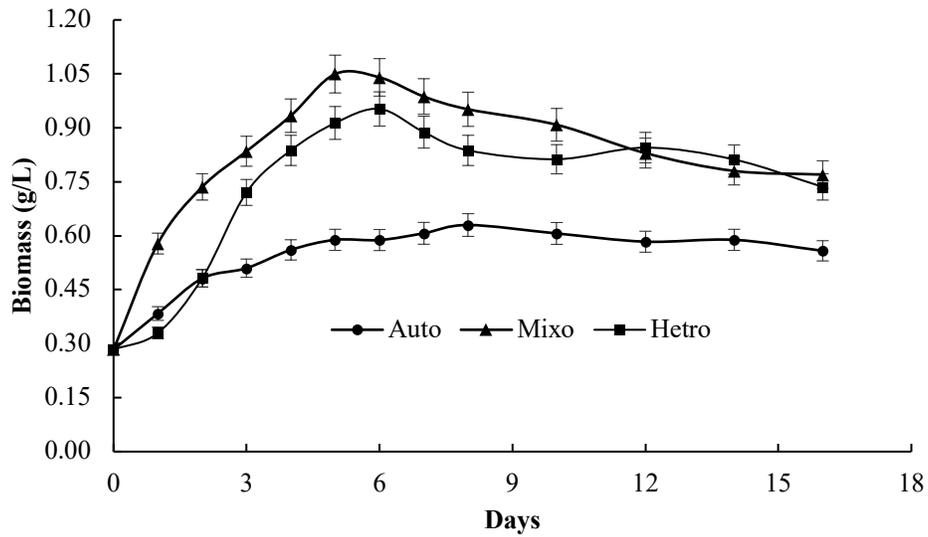


Figure 1: Growth curve of *Chlorella emersonii* following three different modes of culturing (Auto; Autotrophic, Mixo; Mixotrophic and Hetro; Heterotrophic). The maximum biomass was produced after 5 days of culturing in mixotrophic mode. Data represented here are the average of three different trials, with the standard deviation noted as error bars.

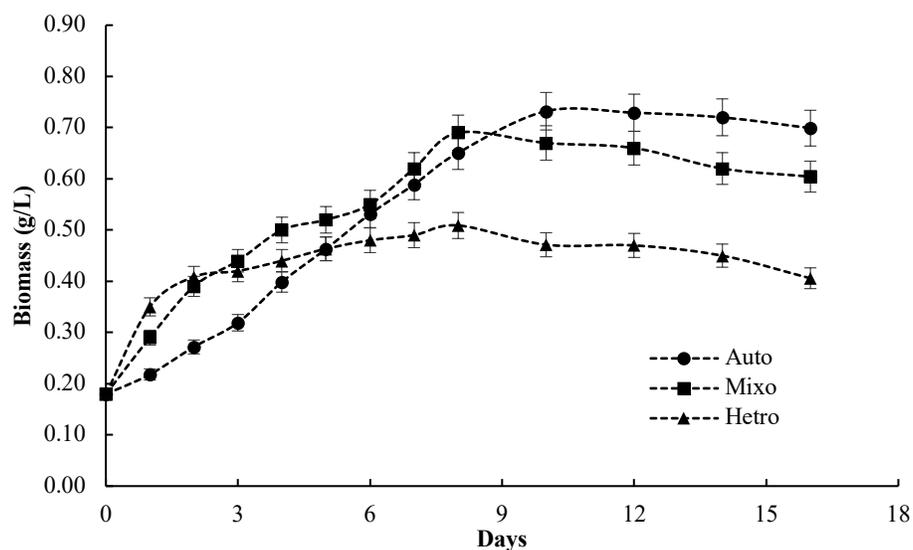


Figure 2: Growth curve of *Pseudokirchneriella subcapitata* following three different modes of culturing (Auto; Autotrophic, Mixo; Mixotrophic and Hetro; Heterotrophic). The maximum biomass was produced after 8 days of culturing in mixotrophic mode and 10 days of culturing in autotrophic mode. The data represented here are the average of three different trials, with standard deviation noted as error bars.

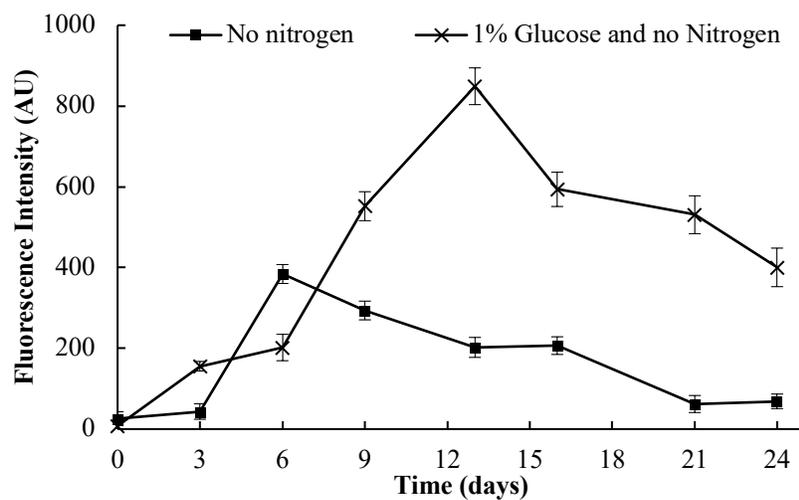


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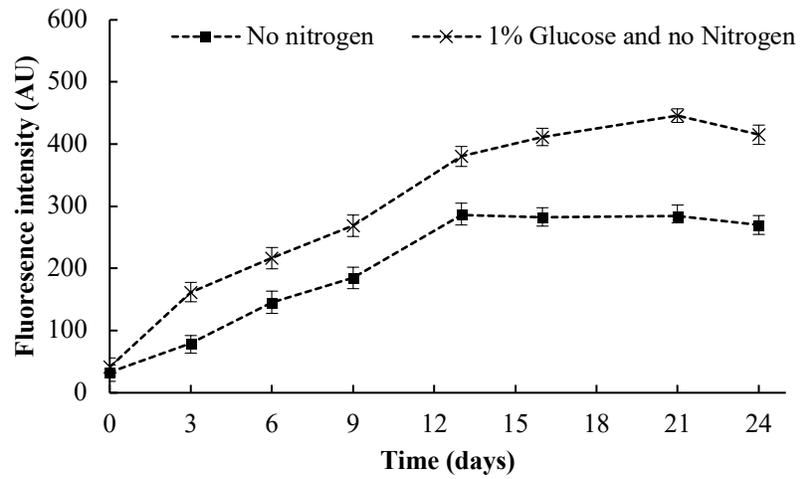


Figure 4: The effect of nitrogen deficient media with, and without, 1% (w/v) glucose on the production of neutral lipids in *Pseudokirchneriella subcapitata* as determined by the Nile Red assay. The data represented here are the average of three different experiments, with five assay values, and the error bars noted are the standard deviation.