Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters

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Abstract

Microalgae have been widely reported as a promising source of biofuels, mainly based on their high areal productivity of biomass and lipids as triacylglycerides and the possibility for cultivation on non-arable land. The isolation and selection of suitable strains that are robust and display high growth and lipid accumulation rates is an important prerequisite for their successful cultivation as a bioenergy source, a process that can be compared to the initial selection and domestication of agricultural crops. We developed standard protocols for the isolation and cultivation for a range of marine and brackish microalgae. By comparing growth rates and lipid productivity, we assessed the potential of subtropical coastal and brackish microalgae for the production of biodiesel and other oil-based bioproducts. This study identified Nannochloropsis sp., Dunaliella salina and new isolates of Chlorella sp. and Tetraselmis sp. as suitable candidates for a multiple-product algae crop. We conclude that subtropical coastal microalgae display a variety of fatty acid profiles that offer a wide scope for several oil-based bioproducts, including biodiesel and omega-3 fatty acids. A biorefinery approach for microalgae would make economical production more feasible but challenges remain for efficient harvesting and extraction processes for some species.

Introduction

Interest in a renewable source of biofuels has recently intensified due to the increasing cost of petroleum-based fuel and the dangers of rising atmospheric CO₂ levels. Among the various candidates for biofuel crops, photosynthetic microalgae have the advantage that they have high growth rates and can be cultured on non-arable land [1,2,3].

At present, microalgae are commercially grown at scale for fatty acid-derived nutraceuticals and as feed and food supply. Significant interest in microalgae for oil production is based on their ability to efficiently convert solar energy into triacylglycerides (TAGs), which can be converted to biodiesel via transesterification reactions [1,4,5]. Oleaginous microalgae are capable of accumulating 20-50% of their dry cell weight as TAGs and potentially have a productivity superior to terrestrial crops used as first generation biofuel feedstock [6]. Theoretical calculations of microalgal oil production (liter/ha) are 10 to 100-fold greater than traditional biodiesel crops such as palm oil [7], corn and soybeans [6,8,9], although large-scale commercial algal oil production has yet to be established. Another major advantage of microalgae over higher plants as a fuel source is their environmental benefits. Despite having to grow in an aquatic medium, microalgae production may require less water than terrestrial oleaginous crops and can make use of saline, brackish, and/or coastal seawater [10,11]. This allows the production of microalgae without competing for valuable natural resources such as arable land, biodiverse landscapes and freshwater. Furthermore, a microalgae-based biofuel industry has tremendous potential to capture CO₂. In high efficiency, large microalgae cultivation systems, the potential capture efficiency of CO₂ can be as high as 99% [12], effectively capturing 1.8 kg of CO₂ per kg of dry biomass [13]. Although CO₂ captured this way into biodiesel will eventually be released upon combustion, this would displace the emission of fossil CO₂ and the remaining biomasses (e.g. ~70% of dry weight) can be fed into downstream carbon sequestration processes. For example, sequestering carbon into hard C-chips (Agri-char) via pyrolysis can be used to improve soil fertility, mitigating climate change by reintroducing durable carbon back into the soil [14], although it is debatable how long this carbon will actually stay in the soil.

Aside from biodiesel production, microalgae are gaining a reputation as “biofactories” due to the varied composition of their biomass. Akin to today’s petroleum refinery, which produces a range of fuels and derivative products, a well-managed and equipped microalgal biorefinery can produce biodiesel and other value-added products such as protein, carbohydrates and a range of fatty acids (FAs). High value omega-3 fatty acids (ω-3) such as...
Evaluation of Oil-Producing Microalgae for Biofuel

Microalgae strain collection and isolation

Microalgae were collected as 10 mL water samples from coastal rock pools, freshwater lakes and brackish (tidal) riverways. After initial cultivation of the mixed cultures with F medium [41] pure cultures were isolated by performing serial dilutions and the use of a micromanipulator (Leica DMIL with Micromanipulator). Strains *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2 originated from the same water sample and were collected from the Brisbane river (27°31′21″S 153°03′32″E; high tide at 10 am in August 2007 on a sunny day). Strain *Tetraselmis* sp. M8 was collected in an intertidal rock pool at Maroochydore (26°39′39″S 153°6′18″E; 12 pm on 6 August 2009). Additional, microalgae strains used in this study were obtained from the Australian National Algae Culture Collection (ANACC, CSIRO) and Queensland Sea Scallops Trading Pty Ltd (Bundaberg, Australia) (Table 1). All primary stock cultures were maintained aerobically in 100 mL Erlenmeyer flasks with constant orbital shaking (100 rpm) at 25°C, under a 12:12 h light/dark photoperiod of fluorescent white light (120 µmol photons m−2 s−1). All cultures except *Chlorella* sp. were grown in seawater complemented with F medium [41]. *Chlorella* sp. was cultured in freshwater complemented with F medium. Primary stock cultures were sub-cultured every 3 weeks to minimize bacterial growth. Non-sterile cultures were used and maintained, as difficulties in maintaining axenic cultures in real production would arise and axenic cultures had been reported to have low biomass productivity, most likely because alga-associated bacteria may assist in nutrient recycling [42]. However, all microalgae cultures were checked during cell counting to ensure that no contamination with other microalgae occurred.

Materials and Methods

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Materials and Methods
Standard protocol for batch culture growth analysis, lipid induction phase and sampling for lipid analysis

A standard protocol was designed to allow direct comparisons of growth rates and lipid productivity between cultures. To standardize inoculum cell densities, cultures were first grown to late logarithmic phase in F medium. Late-log-phase of each culture was determined when daily cell count of the pre-culture revealed a less than 20% increase in cell density. A total of 1 mL of pre-culture in late-log phase was used as inoculum (7 to 9 hours after start of light cycle) for 20 mL seawater (SW) complemented with F medium in 100 mL Erlenmeyer flasks. A minimum of three parallel cultures were grown in conditions as described above. Cell counts were performed on days 0, 2, 4, 6 and 7 post inoculation using a haemocytometer. After day 7, nutrient deprivation to stimulate lipid production was achieved by removal of previous medium by centrifugation (1,200 g, 5 min) and replacement with only SW (without F medium). Cultures were then grown for another 48 h before 4 mL of wet biomass from each replicate was harvested for lipid analyses.

Fatty Acid Methyl Ester (FAME) analyses

Algal cultures (4 mL each) were centrifuged at 16,000×g for 3 min. The supernatant was discarded and lipids present in the algal pellet were hydrolyzed and methyl-esterified by shaking (1,200 rpm) with 300 μL of a 2% H2SO4/methanol solution for 2 h at 80°C; 50 μg of heneicosanoic acid (Sigma, USA) was added as internal standard to the pellet prior to the reaction. A total of 300 μL of 0.9% (w/v) NaCl and 300 μL of hexane was then added and the mixture was vortexed for 20 s. Phase separation was performed by centrifugation at 16,000×g for 3 min. A total of 1 μL of the hexane layer was injected splitless into an Agilent 6890 gas chromatograph coupled to a 5975 MSD mass spectrometer. A DB-Wax column (Agilent, 122–7032) was used with running conditions as described for Agilent’s RTL DBWax method (Application note: 5988–5871EN). FAMEs were quantified by taking the ratio of the integral of each FAME’s total ion current peak to that of the internal standard (50 μg). The molecular mass of each FAME was also factored into the equation. Identification of FAME was based on mass spectral profiles, comparison to standards, and expected retention time from Agilent’s RTL DBWax method (Application note: 5988–5871EN).

DNA isolation and sequencing

Genomic DNA was isolated from all algal species via a phenol-chloroform method [43] on a pellet obtained by centrifugation of 10 mL of algal culture at the late-log phase. DNA amplification from genomic DNA containing a partial 18S ribosomal RNA region was performed by PCR using the following primers: Forward: 5'-GGGTAATTCCAGCTCCAATAGC–3' and Reverse: 5'-GACCATACTCCCCCCCAGAAGC-3'. Briefly, DNA was denatured at 94°C for 5 min and amplified by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min. There was a final extension period at 72°C for 10 min prior to a 4°C hold. The PCR product was isolated using a Gel PCR Clean-Up Kit (Qiagen). For sequencing reactions, 25 ng of PCR product was used as template with 10 pmol of the above primers in separate reactions in a final volume of 12 μL. The samples were then sent to the Australian Genome Research Facility in Brisbane for sequencing. All new data has been deposited in GenBank (Table 1).

Identification of microalgae and phylogenetic analysis

Nucleotide sequences were obtained from the NCBI database based on the BLAST results of each algal sequenced in this study. When sequences from multiple isolates of a species were available, two nucleotide sequences were chosen: (i) highest max score sequence, (ii) highest max score sequence with identified genus and species. Strains Tetrastemis sp. M8, Chlorella sp. BR2 and Nanochloropsis sp. BR2 were isolated by the authors and other strains were obtained from the Australian National Algae Culture Collection (ANACC), CSIRO and Queensland Sea Scallops Trading Pty Ltd (QSST), Bundaberg (Table 1). In total, 22 sequences from the NCBI database and eleven sequences from algae in this study were aligned with the MAFFT [44]. The resulting alignment was then manually inspected for quality and the end gaps trimmed. Phylogenetic analyses of the sequences was performed with PhyML 3.0 [45] using the ML method. Default settings were used, with the exception that 100 bootstraps were used in a nonparametric bootstrap analysis instead of an approximate likelihood ratio test as this is the more commonly used method in recent reports.

Analytical methods

Measurement of nitrate and phosphate levels in the photo-bioreactor was performed using colorimetric assays (API, Aquar-
Growth rate, doubling time and lipid productivity were calculated as follows. The average growth rate was calculated using the equation \( \mu = \frac{\ln(N_y/N_x)}{(t_y-t_x)} \) with \( N_y \) and \( N_x \) being the number of cells at the start \( t_x \) and end \( t_y \) of the growth phase (7 days). Average doubling time \( T_{\text{ave}} \) was calculated using the equation \( T = \frac{(t_y-t_x)}{\log_2 (N_y/N_x)} \) over the growth period of 7 days. The specific growth rate \( \mu_{\text{Max}} \) was calculated between the 2 days of maximum slope on the average cell density x-axis time plot [31,46]. Lipid productivity \( \mu \text{g mL}^{-1} \text{day}^{-1} \) was calculated as total lipid content \( \mu \text{g/mL} \) over the duration of the entire batch culture (laboratory cultures – 9 days, outdoor culture – 12 days).

![Figure 1. Epifluorescent (A, C, E, G, I, K, M, O, Q, S, U) and differential interference contrast (B, D, F, H, J, L, N, P, R, T, V) images of eleven microalgae used in this study. *Chlorella* sp. BR2 (A, B), *Nannochloropsis* sp. BR2 (C, D), *Chaetoceros muelleri* (E, F), *Chaetoceros calcitrans* (G, H), *Pavlova lutheri* (I, J), *Pavlova salina* (K, L), *Isochrysis* sp. (M, N), *Dunaliella salina* (O, P), *Tetraselmis chui* (Q, R), *Tetraselmis sp. MB* (S, T) and *Tetraselmis suecica* (U, V). All images were taken at 100x magnification. Bars represent 20 \( \mu \text{m} \).](doi:10.1371/journal.pone.0040751.g001)
Microscopic analyses
After a lipid induction phase, microalgae cells were stained with 2 μg/mL Nile red (dissolved in acetone; Sigma, USA) for 15 minutes and photographed using a fluorescent Olympus BX61 microscope and an Olympus DP10 digital camera. Differential interference contrast (DIC) and epifluorescent (excitation: 510–550 nm, emission: 590 nm) images were obtained at 1000× magnification with oil immersion.

Mid-scale outdoor cultivation
In order to evaluate the growth performance and lipid productivity of microalgae in a medium-scale outdoor setting, *Tetraselmis* sp. was selected and tested in a 1000 L outdoor photobioreactor built by The University of Queensland’s Algae Biotechnology Laboratory (www.algaebiotech.org) between 20th May 2011 to 1st June 2011 (sunny conditions 22°C–26.5°C). An initial cell density of 1.3×10⁶/mL was cultured in SW + F/2 medium for 10 days (pH 8.8; maintained by the addition of CO₂) followed by 2 days of nutrient starvation (nitrogen measurements were 0 mg/L on day 10). Cell counts were conducted on days 0, 2, 4, 6, 7, 10, 11 and 12 and cultures were checked to ensure that no contamination with other microalgae occurred. To facilitate comparison with laboratory protocols, growth parameters were determined within the first 7 days of culture. At day 10, 4 mL of culture was sampled for lipid analysis.

Statistical analysis
Data for growth rates and lipid productivity was statistically analyzed by one-way analysis of variance (ANOVA) with different microalgae species as the source of variance and growth rate or lipid productivity as dependent variables. This was followed by Bonferroni’s multiple comparisons test where appropriate.

Results
Strain collection, isolation and morphological and phylogenetic characterization of candidate microalgal biofuel strains
Over 200 water samples were collected from diverse aquatic habitats from subtropical regions in Queensland, Australia. These included samples from rock pools in coastal areas at the Sunshine Coast, Moreton Bay, Heron Island, Gold Coast and North Stradbroke Island, as well as freshwater samples from Somerset Dam, Wivenhoe Dam and brackish samples from tidal rivers, including the Brisbane and Logan rivers. Additional microalgae strains were obtained from culture collections at ANACC, CSIRO, and two local isolates from QST, Bundaberg. Visual microscopy (Figure 1) confirmed the isolation of unicellular cultures. Morphological comparisons to other described microalgae suggested that these strains belonged to the genera *Tetraselmis*, *Chlorella*, *Nannochloropsis*, *Dunaliella*, *Chaetoceros*, *Pavlova* and *Isochrysis*.

Nile red staining and growth analysis (Table 2, Figures 1) revealed eleven candidate strains that met the criteria required for biodiesel production (i.e. easy cultivation with no special nutrient requirements, fast growth rate, seawater-strength (35 ppt) salinity tolerance and high lipid production). One promising freshwater culture (*Chlorella* sp. BR2) was also included. Under nutrient-deprived conditions, lipids produced by microalgal cells were observed as bright yellow globules when stained with Nile red and viewed under epifluorescent light (Figure 1).

To specify the identity of the microalgae strains used in our experiments, a partial 18S region of the ribosomal RNA gene was amplified by PCR and sequenced. The obtained sequences were then compared to existing sequences in the NCBI database by the BLAST algorithm (for Genbank accession numbers see Table 1). Homology (sequence identity) searches confirmed a close relationship of the isolated candidate strains *Chlorella* sp. BR2, *Nannochloropsis* sp. BR2 and *Tetraselmis* sp. M8 with other members of the genera *Chlorella* and *Tetraselmis*. *Chlorella* sp. BR2 had a sequence identity of 99% with *Chlorella* sp. Y9 (Genbank Acc. No. JF950558) and *Chlorella vulgaris* CCAP 211/79 (Acc. No. FR863883). *Tetraselmis* sp. M8 shared a sequence identity of 99% with *Tetraselmis suecica* (CS-187) and *Tetraselmis chui* (CS-26). To characterize the diversity of the 11 microalgae strains and their relationship to other microalgae, the obtained sequences from this study were phylogenetically analyzed. The obtained maximum likelihood phylogenetic tree (Figure 2) depicts the placement of each microalgae strain used in this study with chosen BLAST results.

BLAST 18s rRNA sequence comparison of eleven strains from this study to each other and the NCBI database (Figure 2) confirmed the taxonomic classification (suggested by microscopic studies or CSIRO/QSST) in all species based on the maximum score, while revealing high similarity within a species.

Comparison of growth rates, doubling times and cell densities of microalgal strains
To determine and compare growth rates, doubling times and cell densities, all microalgae strains were grown as three side-by-side cultures. After inoculation, an initial lag phase was observed in most cultures, except *Chlorella* sp. BR2, *C. calcitrans*, *C. muelleri* and *I. galbana*, where exponential growth was observed immediately upon inoculation (Figures 3–4). Exponential growth in all cultures occurred till day 7 but for *D. salina*, *P. lutheri*, *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2, a lag phase was observed on day 4. *D. salina* culture remained in lag phase till day 7, while *P. lutheri*, *Chlorella* sp. BR2 and *Nannochloropsis* sp. BR2 resumed growth after day 6.

The highest average growth rate (μave) was found for *P. lutheri* (0.48 μL⁻¹) and *P. salina* (0.45 μL⁻¹) (Table 2), that were significantly (p<0.05) higher to all other species that had a μave of 0.34 μL⁻¹. Specific growth rates (μexp) were also compared with ANOVA, revealing that *T. chui* had the highest μexp at 1.03 μL⁻¹, followed by *Tetraselmis* sp. M8 (0.93 μL⁻¹) and *P. salina* (0.88 μL⁻¹). The fastest doubling times that were significantly different to the others were found for *P. lutheri* (1.45 days) and *Tetraselmis* sp. M8 (outdoor) (1.48 days) (Figure 3), while other microalgae strains had an average doubling time of 2.06 days. Maximum growth occurred during day 0 to day 4.

FAME productivity and fatty acid composition
GC/MS analysis revealed *Nannochloropsis* sp. (6.24 μg mL⁻¹ day⁻¹) to be the highest FAME producer (*ANOVA, P<0.05 in all cases), followed by *D. salina* (4.78 μg mL⁻¹ day⁻¹; *ANOVA, P<0.05 in all cases except Chlorella sp. BR2, 5.9 μg mL⁻¹ day⁻¹) (Table 3; Figure 5). On the other hand, *T. chui* (1.5 μg mL⁻¹ day⁻¹) and *T. suecica* (1.49 μg mL⁻¹ day⁻¹) were the lowest FAME producers. The FA profile of *Nannochloropsis* sp. BR2, *C. calcitrans* and *C. muelleri* consisted predominantly of C16, C16:1 and C20:5 (>70% in total), while *Chaetoceros* strains produced C14 (10.5–11.6%), *Tetraselmis* sp. M8 contained most notably C18:3 (28.9%) and C16 (22.5%), as well as C18:2 (11.7%). *D. salina* and *Chlorella* sp. BR2’s FA profile consisted mostly of C18 (53–57%), unsaturated C18s (37–43%) and unsaturated C20:5 (8–12%) were the main FAs. *I. galbana’s* FA profile was spread across C14 (19%), C16 (16%), C18:1 (22%), C20:3 (22%) and C20:6
(12%). Approximately 44% of *P. salina*’s FAs consist of C14 and C16 FAs, with C20:5 and C22:6 FAs accounting for another 26%. *P. lutheri*’s FA profile consisted largely of C16 (25%), C16:1 (29%), C20:5 (22%) and C14 (11%).

On average, saturated FAs accounted for 40% of the total FAs in this study, consisting mostly of C16 (27.2%), C14 (7.2%) and C18 (6%). Similar amounts (37.4%) of FAs were polyunsaturated and included EPA C20:5 (9.6%), ALA C18:3 (10.4%) and DHA C22:6 (3.9%). Monounsaturated FAs accounted for 21% of the total FAs, consisting mostly of C16:1 (11.7%) and C18:1 (8.3%). *P. salina* was found to have the highest saturated FA (53%), *C. calcitrans* the highest monounsaturated FA (40%), and *D. salina* the highest polyunsaturated FA content (60%). *C. salina* was found to be a major FA (17–37%) in all the strains tested, particularly in *T. chuiana*, *T. suecica* and *Nannochloropsis* sp. BR2. C16:1 FAs were predominantly found in *C. calcitrans*, *C. muelleri* and *Nannochloropsis* sp. BR2, while highest C14 content was found in *P. salina* and *I. galbana*. *I. galbana* also had the highest content of C18:1 FAs, while C18:3 FAs were predominantly found in *D. salina*, *Chlorella* sp. BR2 and *Tetraselmis* sp. M8. *Nannochloropsis* sp. BR2 and *P. lutheri* both had the highest content of EPA C20:5 FAs while DHA C22:6 was predominantly found in *P. salina*. *D. salina* was the only strain found to produce C16:4. It should be noted that due to the small culture volumes in this study certain fatty acids may have remained undetectable.

Outdoor scale-up

The highest lipid productivity for the microalgae strains tested in this study, was measured for *Nannochloropsis* sp. BR2 (Figure 5). However, based on its versatility and resourcefulness of fatty acids, its short doubling times, its ease of handling, and its potentially better lipid extraction efficiency, *Tetraselmis* sp. M8 was identified as a suitable candidate for large-scale cultivation whose FAME profiles would also meet the criteria for a future microalgae biorefinery. To compare laboratory cultivation with larger outdoor cultivation, *Tetraselmis* sp. M8 culture was grown in a 1000 L closed photobioreactor that was inoculated with 20 L of saturated culture. This mid-scale outdoor culture achieved a cell density of 1.6×10⁶ cells mL⁻¹ on day 7, eventually arriving at 2.3×10⁶ cells mL⁻¹ on day 10. Maximum growth rate was found between day 4 and 6 (Table 2) and was similar to average growth rates (0.47 µL⁻¹ and 0.5 µL⁻¹, respectively). The culture entered stationary phase during starvation (after day 10), and cell count did not increase. The mid-scale, outdoor cultivation of *Tetraselmis* sp. M8 achieved a FAME productivity of 4.8 µL mL⁻¹ day⁻¹, consisting mostly of C16 (20.8%), C18 (10.1%) and C18 unsaturated fatty acids (44.6%).

Discussion

In a microalgae-based oil industry, high oil productivity is crucial to achieving commercial feasibility. While growth conditions (e.g. solar radiation and temperature) and culture management are important, the suitable microorganism is fundamental to produce the desired quality and quantity of oil. A suitable microalgae strain must have high lipid productivity, either by possessing a high basal lipid content and/or be inducible to accumulate substantial amounts of lipids. The selected strain should also be easily harvested, amenable to efficient oil extraction and flexible enough to adapt to changing physio-chemical conditions in an outdoor environment [11]. Thus, a locally isolated strain would likely adapt better to local changing environmental conditions and provide a more stable and productive culture.

Sampling at local waterways focused on inter-tidal rock pools, where the microclimate alters frequently between optimal growth conditions and unfavorable conditions (e.g. low nutrients, microoxic conditions, anaerobiosis, low/high light or dry, hot or cold conditions or rapid changes in salinity). Sampling at such locations was considered advantageous because suboptimal conditions would require the algae there to accumulate photo-assimilates such as starch or lipids that have important storage functions in order to survive, thereby increasing the chances of obtaining high lipid content strains [3]. This was followed by an isolation process targeted to select for high growth rate microalgae strains that could be induced to accumulate lipids under nutrient-deprived conditions. Isolation of uniclonal microalgal strains by serial dilution and plating in F-supplemented medium was designed to...
select strains which grew well in F/2 medium, a common nutrient mix used for microalgae culture [31,32,40,41]. Serial dilutions would also select for fast growing strains, which would inevitably dominate a culture. Special attention must be given to ensure that a single fast growing strain does not dominate other potentially high lipid content strains but that may have a slower growth rate. After 48 hours of nutrient deprivation, Nile red staining of the isolated uni-clonal cultures revealed several strains with substantial

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**Figure 2. Maximum likelihood phylogenetic tree of 18S rRNA gene sequences from microalgae used in this study.** Selected sequences from the NCBI database were also included (see Methods for selection criteria). Microalgae analyzed in this study are shown in bold. Numbers represent the results of 100 bootstrap replicates. doi:10.1371/journal.pone.0040751.g002
Figure 3. Growth curves of different microalgae in this study. T. chui, T. suecica, Tetraselmis sp. M8, D. salina, P. salina and Chlorella sp. BR2. Shown are average cell densities ± SD from three biological replicates.

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Figure 4. Growth curves of different microalgae in this study. C. calcitrans, C. muellera, I. galbana, Nannochloropsis sp. BR2, Chlorella sp. BR2, P. lutheri & Tetraselmis sp. M8 (Outdoors). Shown are average cell densities ± SD from two biological replicates (3 replicates for Nannochloropsis sp. BR2 & 1 for Tetraselmis sp. M8 (Outdoors)).

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lipid producing potential. An inherent problem with using Nile red staining was that differences in cell wall structure between species do not allow for equal staining and prevented accurate comparison of lipid productivity between species. For this reason some species with thick cell walls (e.g. some other Nannochloropsis species) that were not included in the subsequent analysis may still have a strong potential as future microalgae crops.

A standard protocol was established to identify the top FAME-producing microalgae strains by comparing the growth rates, FAME productivity and composition of the 11 microalgae strains in this study. Growth rate and FAME productivity data was then compared with other literature (Table 4). It is crucial that any comparison must take into consideration the different growth conditions, culture system and lipid analysis methods (available in Table S1). Both average growth rate (μave) and specific growth rate (μexp) of the 11 analyzed microalgae strains were calculated from cell count growth curves (Figures 3–4). Overall, μave found in the present study were similar or higher than μave published by [36] and [34], aside from [32] which had nearly twice the μave (Table 4). The specific growth rate (μexp) of microalgae is more widely reported in the literature, although many studies only present growth in biomass productivity [11,30,33,35,47]. Comparison with available literature revealed the present study’s overall μexp to be higher than most, with the exception of microalgae from three publications [40,48,49]. The overall high growth rates of this study were observed despite a lack of culture conditions such as air bubbling, CO2 supplementation and longer photoperiods available in other studies (Table 4; Supplementary Table S1). This could be a result of the increased nutrient availability from the F/2 media in comparison with other studies that utilize F/2 media [31,34,36]. Increase in nutrient availability, particularly nitrogen has been documented to increase growth rate [29,30,50], particularly when the nitrogen source in F/2 media, KNO3 is low (0.75 mM). A previous study on Nannochloropsis discovered light intensity to only have a slight effect on growth rates [47], especially during low cell densities (Skye Thomas-Hall, personal communication) and growth rate discrepancies may be due to differences in prior culture history [51]. Ultimately, T. chui and Tetraselmis sp. M8 were found to have the highest μexp. Tetraselmis strains were also the fastest growers in two other studies, [31] and [34]. The growth rate of Nannochloropsis sp. in this study was below average, contrary to findings by Huerlimann et al. [31]. FAME analysis by GC/MS revealed Nannochloropsis sp. BR2 to be the highest TAG producer, followed by D. salina and Chlorella sp. BR2. These three strains have been found to also be high lipid producers in other studies. Rodolfi et al. [11] compared the lipid productivity of 30 microalgae strains and found Nannochloropsis oculata and Chlorella amongst the best producers of lipids, both indoors and outdoors. Likewise, Huerlimann et al. [31] investigated the lipid content of five tropical microalgae and discovered Nannochloropsis sp. to be the highest lipid producer. A strain of Chlorella was similarly found to be a high lipid producer in an evaluation of ten microalgae strains for oil production [33]. Surprisingly, Isochrysis sp., a high lipid producing strain in other studies, [34] and [35], was found to have one of the lowest lipid production rates in this study. Likewise, Tetraselmis strains, top lipid producers in other studies, [31] and [11], produced the least amounts of lipids in this study.

Variations in species strains, growth conditions, experimental design and lipid extraction/analysis methods make quantitative comparisons of lipid productivity and FA content between studies very difficult (Supplementary Table S1). Nevertheless, when compared with Patil et al [35], who similarly analyzed FAME productivity by GC/MS, the total FAME/dry weight (%) of Nannochloropsis sp. BR2 and Tetraselmis sp. M8 was found to be higher, while I. galbana produced the same amount of FAME/dry weight. However, GC/MS obtained FAME productivity of this study was found to be lower than other sources (except for [37]; Table 4) that utilized solvent and gravimetric methods to measure total lipids. This was expected as solvent and gravimetric methods would include FFAs, TAGs and other lipid classes such as polar lipids (e.g. phospholipids and glycolipids) [6], wax esters [52], isoprenoid-type lipids, [53], sterols, hydrocarbons and

Figure 5. FAME levels of microalgae strains grown in batch culture (7 days growth + 2 days starvation by replacement of medium with seawater). Values shown are the averages of three biological replicates ± SD (except Tetraselmis sp.). Different superscripts indicate significant difference at 95% level (ANOVA, Bonferroni’s test; P<0.05). *Mid-scale outdoors culture.

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Table 3. Fatty acid composition in percentage of total FAME of different subtropical Australian microalgae strains after batch culture (7 days growth +2 days starvation).

| Fatty acid | BR2 | BR2 outdoor |
|------------|-----|-------------|
| C12        | 0.2 | 0.1         |
| C14        | 3.5 | 0.9         |
| C15        | 0.4 | 0.1         |
| C16        | 33.0| 37.3        |
| C16:1      | 26.8| 2.5         |
| C16:2      | 0.4 | -           |
| C16:3      | -   | 0.2         |
| C16:4      | -   | -           |
| C17        | 0.4 | 0.1         |
| C18:1      | 6.0 | 13.8        |
| C18:2      | 0.9 | 8.8         |
| C18:3      | 0.4 | 15.1        |
| C18:4      | -   | -           |
| C20        | 0.2 | 0.5         |
| C20:1      | -   | 1.8         |
| C20:4      | 5.9 | 2.6         |
| C20:5      | 18.8| 7.2         |
| Total saturated (%) | 40.7 | 47.9 |
| Total monounsaturated (%) | 32.8 | 18.2 |
| Total polyunsaturated (%) | 26.5 | 34.0 |
| Total FAMEs (µg mL⁻¹) | 56.1 | 13.5 |
| Total FAME/dry weight (%) | 10.6 | 3.2 |
pigments. Furthermore, different growth conditions in other studies such as growth enrichment with carbon dioxide [48,54], increased photoperiods and light intensity [55], different media volumes and larger initial inoculum would explain for the increased lipid productivity in other studies. This is most evident in the study by Rodolfi et al. [11], where similar strains of *P. salina*

### Table 4. Comparison of FAME productivity (µg mL⁻¹ day⁻¹) of present study microalgae with lipid productivity of microalgae species from other references.

| Species                      | Lipid productivity [µg mL⁻¹ day⁻¹] | References                                      |
|------------------------------|-----------------------------------|------------------------------------------------|
| *Nannochloropsis sp. BR2*    | 6.2                               | This study [GC/MS, AG]                          |
| *Nannochloropsis sp.*        | 4.6                               | Huerlimann et al. (2010) [12h]                  |
| *Nannochloropsis sp.*        | 48.0                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Nannochloropsis sp.*        | 37.6                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Nannochloropsis sp.*        | 60.9                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Nannochloropsis oculata*    | 10.0                              | Converti et al. (2009) [34h, CO₂]               |
| *Tetraselmis sp. MB*         | 2.1                               | This study [GC/MS, AG]                          |
| *Tetraselmis sp. MB (outdoor)*| 4.8                               | This study [GC/MS]                              |
| *Tetraselmis sp.*            | 18.6                              | Huerlimann et al. (2010) [12h]                  |
| *Tetraselmis sp.*            | 43.4                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Tetraselmis sp.*            | 10.7                              | Patil et al. (2007) [GC/MS, 24h, CO₂]           |
| *Tetraselmis chui*           | 1.5                               | This study [GC/MS, AG]                          |
| *Tetraselmis chui*           | 27.0                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Tetraselmis suecica*        | 1.5                               | This study [GC/MS, AG]                          |
| *Tetraselmis suecica*        | 36.4                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Dunaliella salina*          | 4.8                               | This study [GC/MS, AG]                          |
| *Dunaliella salina*          | 33.5                              | Takagi et al. (2006)                            |
| *Chaetoceros muelleri*       | 3.3                               | This study [GC/MS, AG]                          |
| *Chaetoceros muelleri*       | 21.8                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Chaetoceros calcitrans*     | 3.2                               | This study [GC/MS, AG]                          |
| *Chaetoceros calcitrans*     | 17.6                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Chaetoceros sp.*            | 16.8                              | Renaud et al. (2002) [12h]                      |
| *Isochrysis galbana*         | 2.0                               | This study [GC/MS, AG]                          |
| *Isochrysis sp.*             | 24.9                              | Renaud et al. (2002) [12h]                      |
| *Isochrysis sp.*             | 12.7                              | Huerlimann et al. (2010) [12h]                  |
| *Isochrysis sp.*             | 37.7                              | Rodolfi et al. (2009) [34h, CO₂]                |
| I. galbana                   | 12.4                              | Patil et al. (2007) [GC/MS, 24h, CO₂]           |
| *Pavlova lutheri*            | 2.0                               | This study [GC/MS, AG]                          |
| *Pavlova lutheri*            | 50.2                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Pavlova salina*             | 2.1                               | This study [GC/MS, AG]                          |
| *Pavlova salina*             | 49.4                              | Rodolfi et al. (2009) [34h, CO₂]                |
| *Pavlova sp.*                | 21.7                              | Patil et al. (2007) [GC/MS, 24h, CO₂]           |
| *Chlorella sp.*              | 3.9                               | This study [GC/MS, AG]                          |
| *Chlorella sp.*              | 7.1                               | Chen et al. (2010) [26h]                        |
| *Chlorella sp.*              | 20.0                              | Converti et al. (2009) [24h, CO₂]               |
| *Chlorella sp.*              | 42.1                              | Rondolfi et al. (2009) [34h, CO₂]               |
| *Chlorella sorokiana*        | 44.7                              | Rondolfi et al. (2009) [34h, CO₂]               |
| *Chlorella sorokiana*        | 1.0                               | Illman et al. (2000) [24h, CO₂]                 |
| *Chlorella vulgaris*         | 5.3                               | Illman et al. (2000) [24h, CO₂]                 |

*Calculated total lipid content (µg mL⁻¹).*

[GC/MS] Values obtained by GC/MS.

[34h] Cultures grown with 34 h light and air.

[12h] Cultures grown with 12 h light and air.

[24h] Cultures grown with 24 h light and air.

*For a full comparison of culturing conditions see Table S1.*

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Nannochloropsis sp. BR2 produced the highest amounts of DHA production in this study were found to be used as dietary supplements. The best candidates for EPA and amounts of biomass. Microalgae possess the potential to produce high lipid productivity, but also suitable FA content. Recommended FAs for biodiesel production include C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 [3,56]. In this study, analyses of FA profiles revealed Nannochloropsis sp. BR2, Chlorella sp. BR2 and Chaetoceros strains (C. calcitrans and C. muelleri) to be the best candidates (Table 3). In addition to having the highest lipid productivity, the recommended FAs for biodiesel accounted for 73.6% of the total FAs in Nannochloropsis sp. BR2, in particular C16 (33%) and C16:1 (26.8%). Huerlimann et al. [31] reported a similar FA composition of Nannochloropsis sp. following nutrient deprivation, while Patil et al. [35] also reported Nannochloropsis sp. to have the highest C16 and C16:1 content. Chlorella sp. BR2 presented slightly lower lipid productivity although having more desired FAs for biodiesel (81.4%). It also had a higher C18 (9.7%) and unsaturated C18 content (39.9%) if compared to Nannochloropsis sp. BR2 or the Chaetoceros strains; making it more desirable for the production of biodiesel with a higher cold filter plugging point (CFFP) for better performance at low temperatures [3]. Both C. calcitrans and C. muelleri are good candidates despite only having medium FAs due to high levels of C14 FAs (10.5% and 11.6% respectively) and recommended FAs for biodiesel (78.9% and 74.5% respectively). The FA content of C. calcitrans was observed in accordance to Lee et al. [34] during low nitrogen conditions, which caused an increase in saturated FAs like C16. D. salina was not considered a suitable candidate for biodiesel due to its high lipid productivity due to high levels of PUFAs (C16:4 – 11.6%, C18:3 – 33.8%). Low levels of PUFAs, as evident in Nannochloropsis sp. and C. calcitrans are desired for biodiesel production as it reduces the need for treatments such as catalytic hydrogenation. Nannochloropsis sp. BR2, C. calcitrans and C. muelleri also exhibited C20:5 (EPA) (18.8%, 12.7% and 14% respectively) which would allow for a biofinery approach to biodiesel production. It should be noted, however, that microalgae biodiesel is likely to be first used as a drop-in fuel in the future which would allow to achieve blends with the desired fuel properties from most microalgae species.

Commercially feasible production of microalgae biodiesel would require a biofinery approach to produce biodiesel as well as other value-added products such as FAs and protein-rich biomass. Microalgae possess the potential to produce high amounts of ω-3 FAs such as EPA (C20:5) and DHA (C22:6) that are used as dietary supplements. The best candidates for EPA and DHA production in this study were found to be Nannochloropsis sp. BR2 and the Pavlova strains (P. salina and P. lutheri). Overall, Nannochloropsis sp. BR2 produced the highest amounts of ω-3 FAs on account of its high overall lipid and EPA content (18.8%). P. lutheri exhibited the highest proportional content of EPA (21.8%), while Isochrysis sp. had the highest DHA content (11.8%). The ω-3 FA contents of Nannochloropsis sp. and the Pavlova strains were comparable to previously published values [31,35,37].

The use of a nutrient starvation phase to improve TAG productivity (particularly C16:0 and C16:1) for biodiesel production was successful as C16 and C16:1 FAs were found to be the predominant FAs in the present study. During nutrient limiting conditions, unsaturated FAs are consumed as an energy source and saturated FAs are accumulated [58]. The increase of the % of saturated and monounsaturated FAs during starvation have been well documented in literature for several other species [34,59,60]. While this may prove useful for biodiesel production, the reduction in PUFAs is a problem for ω-3 FA production that has been documented [31,34]. Nevertheless, EPA and DHA contents have been reported to remain consistent despite changes in nutrient level for T. tetraethole [40], which may explain the high levels of PUFAs observed in Tetraselmis sp.

In a 1000 L-outdoor setting, Tetraselmis sp. M8 was found to have an increased µmax despite a longer lag phase. Cell density achieved by outdoor grown Tetraselmis sp. M8 was similar to other large-scale cultures of Tetraselmis [61]. FAME productivity and composition were also analyzed, which revealed a near tripling of FAME productivity as well as altered FA composition. High amounts of C16:2, C18:2, C18:3 previously detected in laboratory-grown Tetraselmis sp. M8 was found reduced, while higher amounts of recommended FA for biodiesel (particularly C14, C18 & C18:1) were present. The increase in FAME productivity and desirable FA composition of Tetraselmis sp. M8 in a mid-scale setting demonstrates that the microalgae isolation and selection technique used in this study can lead to the identification of microalgae strains with potential for large-scale cultivation. Additional factors to be considered for large-scale production include harvesting and oil extraction properties of different microalgae. For example, we noticed that our Tetraselmis strains may lose their flagella during stress conditions, resulting in rapid settling that allows easy harvesting/dewatering. Small microalgae, such as Nannochloropsis sp., on the other hand may instead be harvested by froth flotation or other techniques, but our results indicate that Nile red staining and lipid extraction may be compromised by thick cell walls in this strain.

**Supporting Information**

Table S1 Comparison of FAME productivity (µg mL⁻¹ day⁻¹) of present study microalgae with lipid productivity of microalgae species from other references (including a full comparison of culturing conditions).

(PDF)

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**Author Contributions**

Conceived and designed the experiments: DKYL SG MT ESZB SRTH YL. PMS. Performed the experiments: DKYL SG MT ESZB SRTH. Analyzed the data: DKYL SG MT ESZB SRTH HS YL. Contributed reagents/materials/analysis tools: MT HS PMS. Wrote the paper: DKYL HS MT YL. PMS.
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