Bioprocess engineering and downstream process optimisation of microalgae for biomass production

Sourabh Surinder Garg

Masters of Biotechnology

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2014

School of Agriculture and Food Sciences
ABSTRACT

Microalgae have been considered a promising option to provide food, feed and biofuel based on their high areal productivity and their ability to be cultured on non-arable land with different water sources (fresh, brackish or seawater). This research thesis work evaluated different cultivation systems for growing the marine microalga *Tetraselmis* sp. (M8) and optimised dispersed air flotation (DiAF) as a separation and harvesting method using the research facilities in the Algae Biotechnology Laboratory and Mineral Processing Laboratory at the University of Queensland’s St. Lucia campus.

Current algae cultivation systems are threatened by contamination with other algae or algal grazers resulting in the need for significant improvement in cultivation and harvesting systems. So far, not much work has been carried out on flotation methods, a process widely used for recovery of particles in mining, for harvesting of marine microalgae. To address these issues, we have developed an efficient two-stage cultivation system using the marine microalga *Tetraselmis* sp. M8. This hybrid system combines exponential biomass production in positive pressure air lift-driven bioreactors with a separate synchronised high lipid induction phase in nutrient deplete open raceway ponds. A comparison to either bioreactor or open raceway pond cultivation systems suggests that this process potentially leads to significantly higher productivity of algal lipids that can serve as a feedstock for biodiesel production. Nutrients are only added to the closed bioreactors and open raceway ponds have turnovers of only a few days, thus avoiding the critical issue of contamination.

Once the cultivation of marine species was optimised, the focus of this study was directed towards optimisation of DiAF method. An initial study was carried out to harvest freshwater microalgae (*Chlorella* sp. BR2) and marine microalgae (*Tetraselmis* sp. M8) from dilute culture with and without a collector, tetradecyl trimethylammonium bromide (C\textsubscript{14}TAB). The surface hydrophobicity of microalgae was measured by using a modified adherence-to-hydrocarbon method. If no collector was added, BR2 showed high hydrophobicity, and its flotation tests in a mechanically agitated cell produced an algal concentrate with an enrichment ratio of 13.5 and 90.3% algae recovery. The natural hydrophobicity of M8 was low, so was its flotation recovery (6.4%). Addition of C\textsubscript{14}TAB improved M8 recovery to 71.1% but with a low enrichment ratio of 3.4 times.

Overall, the flotation performance correlated well with algal hydrophobicity. In search of more effective collectors for marine algae flotation, the hydrophobicity of M8 in aqueous
solutions of varying surfactant type and concentration and pH was measured. It was found that addition of dodecylammonium hydrochloride (DAH) at 25 ppm and pH 6 significantly enhanced the hydrophobicity of M8. Subsequent flotation results confirmed that at this chemical condition, M8 enrichment ratio was increased to 6.6 with 80.5% algae recovery. Further improvement was achieved by using a Jameson cell with relatively small air bubbles. The Jameson flotation for M8 gave an enrichment ratio of 11.4 times with 97.4% algae recovery.

To make the process more cost effective it was necessary to carry out DiAF as close to the growth medium's natural pH as possible. On testing separation of marine microalga M8 from seawater with various 12-carbon chain collectors, such as dodecyl pyridinium chloride (DPC), N-dodecylpropane-1,3-diamine hydrochloride (DN2), dodecyl amine hydrochloride (DAH), and sodium dodecyl sulphate (SDS) were added. DPC at natural growth medium pH (9.5) outperformed DAH, DN2 and SDS. For DPC, the use of a Jameson cell further improved the flotation performance from 16 times to a final enrichment ratio of 23 times, with over 99% marine microalgae recovered.

The present study helped to refine the flotation process and led to a deeper understanding on how marine microalgae can be harvested by using DiAF. During the study it was also found that hydrophobicity plays a key role in microalgae recovery. Different microalgae have different natural hydrophobicity. Generally, collector chemicals are required to increase the efficiency of the flotation process. However, the use of chemicals restricts the application of harvested biomass and makes it unfit for feed or food purposes. During the hydrophobicity testing of different microalgae, some were found to possess naturally higher hydrophobicity. Hence a comparative study was needed to identify the microalgal species that can be flotated without the addition of chemicals. The study showed that freshwater strains *Chlorella* sp. BR2 and *Scenedesmus* sp. NT8c possess hydrophobicity values of up to 28.85% and 23.88% which indicated that they can be harvested effectively without the addition of collector chemicals. The DiAF study showed that 85.69% and 66.29% of *Chlorella* sp. BR2 and *Scenedesmus* sp. NT8c could be recovered using a mechanical cell with enrichment ratios of 21.98 and 17.43. Although these values are lower than those obtained with collectors, the harvested biomass would be suited for animal feed, while the remaining uncollected algae can be used as a continued culture. In conclusion, this study has demonstrated that DiAF is a suitable technique for effective and large-scale harvesting of both freshwater and marine microalgae.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the General Award Rules of The University of Queensland, immediately made available for research and study in accordance with the Copyright Act 1968.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.
Publications during candidature

Peer-reviewed papers


Conference abstracts


Publications included in this thesis

- Publication citation – incorporated as part of chapter 2.


<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Garg S. (Candidate)</td>
<td>Wrote and edited paper (30%)</td>
</tr>
<tr>
<td>Author Sharma KK.</td>
<td>Wrote and edited paper (30%)</td>
</tr>
<tr>
<td>Author Yan Li.</td>
<td>Wrote and edited paper (5%)</td>
</tr>
<tr>
<td>Author Malekizadeh A.</td>
<td>Wrote paper (5%)</td>
</tr>
<tr>
<td>Author Schenk P.M.</td>
<td>Wrote and edited paper (30%)</td>
</tr>
</tbody>
</table>

- Publication citation – incorporated as part of chapter 3


<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Lim D.K.Y.</td>
<td>Designed experiment (50%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (55%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (60%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (65%)</td>
</tr>
<tr>
<td>Author Garg S. (Candidate)</td>
<td>Designed experiment (20%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (35%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (20%)</td>
</tr>
<tr>
<td></td>
<td>Wrote paper (5%)</td>
</tr>
<tr>
<td>Author Timmins M.</td>
<td>Performed experiment (5%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (15%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (5%)</td>
</tr>
<tr>
<td>Author Zhang E.S.B.</td>
<td>Designed experiment (10%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (5%)</td>
</tr>
<tr>
<td>Author Thomas-Hall S.R.</td>
<td>Wrote and edited paper (5%)</td>
</tr>
<tr>
<td>Author Schuhmann H.</td>
<td>Data analysis and interpretation (5%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (5%)</td>
</tr>
<tr>
<td>Author Schenk P.M.</td>
<td>Designed experiment (20%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (15%)</td>
</tr>
</tbody>
</table>
- Draft citation – incorporated as part of chapter 3

**Garg S., Narala R.R., Thomas-Hall, Sharma KK., Deme M., Li Y., Schenk, P.M.**
Comparison of microalgae cultivation in photobioreactor, open raceway pond and a two-stage hybrid system

<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Garg S. (Candidate)</td>
<td>Designed experiment (40%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (40%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (40%)</td>
</tr>
<tr>
<td></td>
<td>Wrote paper and edited paper (30%)</td>
</tr>
<tr>
<td>Author Narala R.R.</td>
<td>Designed experiment (40%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (40%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (40%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (30%)</td>
</tr>
<tr>
<td>Author Sharma KK</td>
<td>Performed experiment (5%)</td>
</tr>
<tr>
<td>Author Thomas-Hall S.R.</td>
<td>Data analysis and interpretation (5%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (5%)</td>
</tr>
<tr>
<td>Author Miklos Deme</td>
<td>Designed experiment (5%)</td>
</tr>
<tr>
<td>Author Li Y</td>
<td>Performed experiment (10%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (5%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (5%)</td>
</tr>
<tr>
<td>Author Schenk P.M.</td>
<td>Designed experiment (15%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (5%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (30%)</td>
</tr>
</tbody>
</table>

- Publication citation – incorporated as a chapter 4


<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Garg S. (Candidate)</td>
<td>Designed experiment (70%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (100%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (60%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (70%)</td>
</tr>
<tr>
<td>Author Yan Li.</td>
<td>Data analysis and interpretation (5%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (5%)</td>
</tr>
<tr>
<td>Author Liguang Wang.</td>
<td>Designed experiment (25%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (25%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (20%)</td>
</tr>
<tr>
<td>Author Schenk P.M.</td>
<td>Designed experiment (5%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (5%)</td>
</tr>
</tbody>
</table>
• Publication citation – incorporated as a chapter 5


<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Garg S. (Candidate)</td>
<td>Designed experiment (75%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (100%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (60%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (70%)</td>
</tr>
<tr>
<td>Author Liguang Wang.</td>
<td>Designed experiment (20%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (25%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (20%)</td>
</tr>
<tr>
<td>Author Schenk P.M.</td>
<td>Designed experiment (5%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (15%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (10%)</td>
</tr>
</tbody>
</table>

• Submitted Paper – incorporated as a chapter 6


<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author Garg S. (Candidate)</td>
<td>Designed experiment (75%)</td>
</tr>
<tr>
<td></td>
<td>Performed experiment (100%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (60%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (70%)</td>
</tr>
<tr>
<td>Author Liguang Wang.</td>
<td>Designed experiment (20%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (25%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (20%)</td>
</tr>
<tr>
<td>Author Schenk P.M.</td>
<td>Designed experiment (5%)</td>
</tr>
<tr>
<td></td>
<td>Data analysis and interpretation (15%)</td>
</tr>
<tr>
<td></td>
<td>Wrote and edited paper (10%)</td>
</tr>
</tbody>
</table>
Contributions by others to the thesis

Critical conceptions, experimental design interpretation of research data; drafting significant parts of the work or critically revising it so as to contribute to the interpretation and were assisted by Prof Peer Schenk, Dr Liguang Wang and Dr Yan Li. To carry out non-routine technical work help was obtained from Kalpesh Sharma, Rakesh Reddy Narala, Dr Simon Tannock and Dr Skye Thomas-Hall.

Statement of parts of the thesis submitted to qualify for the award of another degree

None
Acknowledgements

First and foremost I would like to sincerely thank my supervisor Prof. Peer Schenk for permitting me to explore my ideas and try to figure things out for myself, at the same time making sure that his expert advice and guidance was always there when I needed it. I would also like to thank my associate supervisors Dr. Liguang Wang for introducing me to the amazing flotation technology and helpful advice and Dr. Yan Li for providing me with insights on experimental planning and good time management.

I would also like to especially thank Kalpesh Sharma, Rakesh R. Narala, Skye Thomas-Hall, Simon Tannock, Ali Malekizadeh, Faruq Ahmed, David Lim, Urvi Parekh and all other members of the Schenk group for their friendship, reality checks, support and advice throughout the thesis.

I owe a special thank you to Bhavya Vora, Kasturee Jagirdar and Yash Chhabra without their support, help and understanding I would not have been able to finish this PhD.

Finally, a special thanks to my family for their undying love, support, patience with my bad temper and a very special thank you to my partner Forum, for her endless patience, care and support throughout my PhD.
Keywords
Marine and Freshwater microalgae, various cultivation systems, harvesting and dewatering, froth flotation, hydrophobicity, surfactant solubility, bubble size, algae recovery, enrichment ratio, Jameson cell.

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060599, Microbiology not elsewhere classified, 60%
ANZSRC code: 090404, Membrane and Separation Technologies, 30%
ANZSRC code: 060701, Phycology, 10%

Fields of Research (FoR) Classification

FoR code: 0605, Microbiology, 60%
FoR code: 0904, Chemical engineering, 30%
FoR code: 0607, Phycology, 10%
2 (B): Comparison of microalgae cultivation in photobioreactor, open raceway pond and a two-stage hybrid system ................................................................. 72

Background ........................................................................................................... 72

Key findings: ........................................................................................................... 72

Comparison of microalgae cultivation in photobioreactor, open raceway pond and a two-stage hybrid system ........................................................................ 73

Abstract .................................................................................................................. 73

Introduction ............................................................................................................. 73

Materials and Methods .......................................................................................... 75

Algae culturing and analyses ................................................................................... 75

Culture scale-up and monitoring ............................................................................ 76

Results ....................................................................................................................... 77

Design and construction of pilot-scale photobioreactor and raceway ponds ......... 77

Outdoor photobioreactor cultivation ...................................................................... 79

Open raceway pond cultivation .............................................................................. 80

Two-stage hybrid cultivation system ...................................................................... 80

Comparison of individual open pond or PBR cultivation with two-stage hybrid cultivation system .................................................................................... 83

Discussion ............................................................................................................... 85

Acknowledgements ............................................................................................... 87

References .............................................................................................................. 87

3. Flotation of marine microalgae: Effect of algal hydrophobicity ............................. 91

Background ............................................................................................................ 91

Key findings: .......................................................................................................... 91

Flotation of marine microalgae: Effect of algal hydrophobicity .............................. 92
Abstract .................................................................................................................. 92
Highlights .............................................................................................................. 92
Keywords ................................................................................................................ 92
Introduction .......................................................................................................... 92
Methods ................................................................................................................ 94
Cultivation of algae ................................................................................................. 94
Dispersed air flotation test ...................................................................................... 94
Hydrophobicity test ................................................................................................. 95
Results and discussion .......................................................................................... 95
Changing collector dosage ...................................................................................... 95
Modifying salinity ................................................................................................... 98
Conclusions ........................................................................................................... 100
Acknowledgements .............................................................................................. 100
References ............................................................................................................ 100
4. Effective harvesting of low surface-hydrophobicity microalgae by froth flotation .... 102
Background .......................................................................................................... 102
Key findings: .......................................................................................................... 102
Effective harvesting of low surface-hydrophobicity microalgae by froth flotation .... 103
Abstract .............................................................................................................. 103
Highlights ............................................................................................................ 103
Keywords .............................................................................................................. 103
Introduction ......................................................................................................... 104
Methods .............................................................................................................. 105
5. Flotation separation of marine microalgae from aqueous medium

Background

Key findings:

Flotation separation of marine microalgae from aqueous medium

Abstract

Introduction

Materials and Methods

Cultivation of algae

Hydrophobicity test

Dispersed air flotation

Collector solubility

Froth stability

Results and Discussion

Flotation of microalgae at natural pH

Flotation of microalgae at pH 6

Jameson cell gave better flotation performance than mechanical cell

Conclusions
Acknowledgements ........................................................................................................ 126

Appendix A. Supplementary data ................................................................................ 126

References ................................................................................................................... 127

6. Chemical-free froth flotation of more hydrophobic microalgae ............................... 130

Background ................................................................................................................ 130

Key findings .................................................................................................................. 130

Chemical free froth flotation of hydrophobic microalgae ........................................... 131

Introduction ............................................................................................................... 131

Materials and Method ............................................................................................... 132

Algal culture and characterisation ................................................................................. 132

Modified BATH test for hydrophobicity measurement .................................................. 132

Froth flotation ............................................................................................................. 133

Recultivation ................................................................................................................. 133

Results ....................................................................................................................... 133

Hydrophobicity testing using hexane .......................................................................... 133

Chemical free froth flotation of D. salina ........................................................................ 135

Re-cultivation of microalgae ........................................................................................ 136

Conclusion ................................................................................................................ 137

Reference ................................................................................................................ 137

7. Conclusion and future prospects ............................................................................... 139

Conclusion ............................................................................................................... 139

Future prospects ....................................................................................................... 142

Appendix .................................................................................................................... 144
LIST OF FIGURE

Literature Review

A: Commercial large-scale cultivation for biofuels feed stocks

Figure 1: Tubular photo-bioreactor (left) and raceway pond (right); at the Algae Biotechnology Laboratory, University of Queensland ................................................................. 7

B: Critical analysis of current microalgae dewatering techniques

Figure 2: Three-cornered interactive system affecting flotation and particle-bubble interaction. 12

Figure 3: Division of collectors ...................................................................................................... 13

Figure 4: Effect of ionic strength on the removal of Scenedesmus quadricauda when SDS was used as the collector ..................................................................................................................... 16

Figure 5: Effect of ionic strength on the removal of Chlorella sp. when SDS was used as the collector ......................................................................................................................................... 16

Figure 6. Overview of microalgae harvesting techniques. ......................................................................................................................... 22

Figure 7. Interaction between different harvesting techniques. ................................................. 32

Chapter 2

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

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. M8 (S, T) and Tetraselmis suecica (U, V). All images were taken at 100x magnification. Bars represent 20 mm. ........................................................................................................... 52

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. ............................................................... 54

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 6 SD from three biological replicates ........................................................................................................ 55
Figure 4. Growth curves of different microalgae in this study. C. calcitrans, C. muelleri, 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)). ................................................ 55

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.¹). Different superscripts indicate significant difference at 95% level (ANOVA, Bonferroni’s test; P<0.05). ¹Mid-scale outdoors culture. ...............57

B: Comparison of microalgae cultivation in photobioreactor, open raceway pond and a two-stage hybrid system

Figure 1. Design and specifications of pilot-scale (A) tubular photobioreactor (1:35 scale) and (B) two open raceway ponds (1:75 scale). Mixing was achieved by aeration with pressurized air using an airlift (aeration disc with 6,000 exit holes for the PBR and a single exit point at a lowered section of the raceway ponds). .................................................................................................................................................. 78

Figure 2. Photograph of pilot-scale two-stage microalgae cultivation system. Individual modules were used for single testing of photobioreactor or open ponds. .............................................................................................................79

Figure 3. Two Nile red-stained samples (10x) before (left) and after nutrient deprivation stress at the time of harvesting (right). ..................................................................................................................................................79

Figure 4. Monitoring of photobioreactor cultivation for 33 days. (A) Daily global solar irradiance. (B) Minimum and maximum air temperature recorded for the respective day. (C) Cells (10⁴) per mL. White and red colours represent the start and finish of each cycle, respectively.............81

Figure 5. Monitoring of raceway pond cultivation for 33 days. (A) Daily global solar irradiance. (B) Minimum and maximum air temperature recorded for the respective day. (C) Cells (10⁴) per mL. White and red colours represent the start and finish of each cycle, respectively.............82

Figure 6. Observations of photobioreactor for two-stage cultivation for 35 days. (A) Daily global solar irradiance. (B) Minimum and maximum air temperature recorded for the respective day. (C) Cells (10⁴) per mL. White and red colours represent the start and finish of each cycle, respectively........................................................................................................................................83

Figure 7. (A) Growth rates, and (B) areal biomass productivities for different cultivation systems. Different letters show statistically significant differences. (P<0.05) ............................................. 84

Figure 8. Areal biomass productivity normalized to daily global solar exposure for different cultivation systems. Different letters show statistically significant differences. (P<0.05) .............86
Chapter 3
Flotation of marine microalgae: Effect of algal hydrophobicity

Figure 1. Kinetics of flotation of freshwater microalgae BR2 (a) and of marine microalgae M8(b) at pH 9.5. The lines represent the best fits of Eq. (4) to the experimental flotation recovery data, with cmax being 1 for BR2. 

Figure 2. Flotation recovery and surface hydrophobicity of (a) freshwater microalgae BR2 with 3 ppm C14TAB at various NaCl concentrations and incubation times and (b) marine microalgae M8 at various C14TAB concentrations (3, 30, and 80 ppm) in freshwater and seawater. Each flotation test lasted for six minutes, and the overall flotation recovery was reported.

Chapter 4
Flotation separation of marine microalgae from aqueous medium

Figure 1. (a) Algal surface hydrophobicity (H), (b) flotation recovery (Y), and (c) enrichment ratio (ER) for harvesting of marine microalga Tetraselmis sp. M8 at different dosages of C14TAB collector at pH 9.5 and DAH at pHs 4, 6 and 9.5. The error bars represent typical sample standard deviation.

Figure 2. (a) Direct comparison of DAH and C14TAB as collector for mechanical flotation performance of Tetraselmis sp. M8; (b) Enrichment ratio versus flotation recovery for microalgal cultures Tetraselmis sp. M8 using mechanical cell and Jameson cell with 25 ppm DAH at pH 6. Lines are drawn to guide the eye, and are expected to follow towards a recovery limitation of 100% at which the enrichment ratio would be 1.

Figure 3. Flotation recovery (Y) as a function of algal surface hydrophobicity (H) for M8 with using (a) C14TAB and (b) DAH. The straight line represents the best linear fit to all the data points shown in this subset.

Chapter 5
Flotation separation of marine microalgae from aqueous medium

Figure 1. Effect of collector type on flotation performance shown jointly by enrichment ratio (ER) and algal recovery (Y). The flotation tests were carried out at natural pH (9.5) at two collector dosages: a) 10, and b) 25 ppm. Vertical and horizontal error bars represent the standard errors of enrichment ratio and algal recovery for mean values from three replicates, respectively. Data of DAH were adapted from Garg et al 2014.
Chapter 6

Chemical-free froth flotation of more hydrophobic microalgae

Figure 1. Algae biomass recovery for different microalgae using flotation method..................134

Figure 2. Volume of water recovered with algae by flotation method.................................135

Figure 3. Enrichment ratios of different microalgae by flotation........................................135

Figure 4. Chlorophyll A and B concentration in control and hydrophobicity test samples......136

Figure 5. Overall biomass of *D. salina* during the start each cultivation cycle from tailings and at
the end of the cultivation phase. ..............................................................................................136

Figure 6. Overall biomass of *Chlorella* sp. (left) and *Scenedesmus* sp. (right) during the start of
each cultivation cycle from tailings and at end of the cultivation phase..................................137
LIST OF TABLES

Literature Review
A: Commercial large-scale cultivation for biofuels feed stocks

Table 1: Comparison of various algae cultivating methods.................................................................8

Table 2: Algae recovery with Chitosan (surface modifier), using different collectors ...............17

B: Critical analysis of current microalgae dewatering techniques

Table 1. Examples of various flocculation studies that have been used to harvest microalgae. .................................................................................................................................................24

Table 2. Examples of various flotation studies that have been used to harvest microalgae. .27

Table 3. Examples of various filtration studies that have been used to harvest microalgae. ..29

Table 4. Cost of harvesting 10,000L of Chlorella sp. with different harvesting techniques .......31

Chapter 2
A: Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters

Table 1. Sources and 18S rRNA sequence accessions of microalgae strains used in this study.49

Table 2. Growth rate analysis of eleven microalgae strains during growth phase (7 days) of batch culture.................................................................................................................................................51

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)..........................57

Table 4. Comparison of FAME productivity (mg mL$^{-1}$ day$^{-1}$) of present study microalgae with lipid productivity of microalgae species from other references. .........................................................60

B: Comparison of microalgae cultivation in photobioreactor, open raceway pond and a two-stage hybrid system

Table 1. Comparison of various algae cultivating methods considering data from the present and previous studies ..................................................................................................................87
**List of Abbreviation**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mM</td>
<td>Mill molar</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>kWh</td>
<td>Kilowatts hour</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>µm</td>
<td>Micrometre</td>
</tr>
<tr>
<td>cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>m³</td>
<td>Cubic metre</td>
</tr>
<tr>
<td>K'</td>
<td>Growth rate</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>ppt</td>
<td>Parts per thousand</td>
</tr>
<tr>
<td>rpm</td>
<td>Rounds per minute</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>HRP</td>
<td>High rate pond</td>
</tr>
<tr>
<td>PBR</td>
<td>Photobioreactor</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>DAF</td>
<td>Dissolved air flotation</td>
</tr>
<tr>
<td>DiAF</td>
<td>Dispersed air flotation</td>
</tr>
<tr>
<td>H</td>
<td>Hydrophobicity</td>
</tr>
<tr>
<td>Y</td>
<td>Recovery</td>
</tr>
<tr>
<td>ER</td>
<td>Enrichment ratio</td>
</tr>
<tr>
<td>WR</td>
<td>Water recovery</td>
</tr>
<tr>
<td>WRR</td>
<td>Water rejection rate</td>
</tr>
<tr>
<td>GHG</td>
<td>Greenhouse gas</td>
</tr>
<tr>
<td>TSS</td>
<td>Total suspended solids</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>Sodium nitrate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>C₁₄TAB</td>
<td>Tetradecyl trimethylammonium bromide</td>
</tr>
<tr>
<td>DN₂N</td>
<td>Dodecyl propane 1,3-diamine hydrochloride</td>
</tr>
<tr>
<td>DPC</td>
<td>Dodecyl pyridinium chloride</td>
</tr>
<tr>
<td>DAH</td>
<td>Dodecyl amine hydrochloride</td>
</tr>
<tr>
<td>ECF</td>
<td>Electrocoagulation flocculation</td>
</tr>
</tbody>
</table>
GENERAL INTRODUCTION AND RESEARCH QUESTION

Over-exploitation and unsustainable practices of the oil industry has increased oil prices, aiding to the global political tension [1, 2] and creating various environmental problems. Furthermore, combustion of fuels (oil and its by-products) release harmful gases (mainly CO, CO₂, oxides of nitrogen and sulphur) into the environment, leading to greenhouse gas effect and causing many other health problems [3]. Thus, to prevent climatic changes, immediate reduction in the emission of greenhouse gases is required. Biological mitigation of CO₂ using trees could be one of the ways that can help to reduce greenhouse effect [1]. Developing CO₂ free fuel such as “biohydrogen” or CO₂ neutral fuels such as bioethanol, biodiesel, biomethane [3] could be another method. Development of CO₂ free or neutral fuels will help decrease oil consumption as well as the dependency on major oil producing countries [4]. There have been a lot of research regarding using biohydrogen as a future fuel for transportation, but their results so far have not been promising [5]. Therefore, as the continuous use of fossil fuel becomes unsustainable, with supplies depleting and pollution levels increasing [1], there is a desired need of alternative and sustainable fuel, to which, biofuels could be the immediate solution.

Microalgae have shown the highest potential for producing lipids which can be eventually converted into biodiesels [1, 5]. Currently, popular species which used for biodiesel production are Botryococcus braunii, Nannochloropsis sp., Neochloris oleoabundans, Schizochytrium sp., Tetraselmis sp., Scenedesmus [5, 14, 15]. Microalgae have numerous advantages over the higher plants (crops) used for biodiesel production.

Under the Aquatic Species Program launched by U.S. National Renewable Energy Laboratory, experiments were carried using two 1000 m² high growth rate ponds. The results from test concluded that it is technically feasible to use microalgae for the low-cost production of biodiesel [6]. Unfortunately, despite the various advantages, current lipid yields from microalgae are 10 – 20 times lower than the projected theoretical values [7], making it difficult for companies to produce biodiesel commercially. Thus far, the lowest production cost has been achieved by Seambiotic Ltd in Israel at US$126 – 209 per barrel [3]. More lab scale research and pilot studies have to be carried out in order to increase microalgae production yields while reducing production cost. However, species selection by itself is not only significant, as an engineering aspect is also required for the mass culturing of microalgae. The three main steps in the microalgae-based biofuel process are cultivation of microalgae, biomass harvesting, and oil extraction from microalgae.
There are a few of bottlenecks to hold back the development of the microalgae biofuels, two of them are:

- Commercial large-scale cultivation for the biofuels feed stocks.
- Efficient and cheap harvesting process.

My PhD study will focus on the optimised cultivation model and development of effective harvesting method to address these issues. Issues will be addressed by achieving following 3 aims.

**Aim 1 Algae Screening, Comparison and optimisation of two outdoor mass cultivation systems for microalgae**

A. Lab scale screening of several species of the algae from the collection for large scale cultivation.

B. Based on the data (A), microalgae strains will be selected and cultivated in three different kinds of system. These systems are

   i. A **closed system** made up of a 1,000 L photobioreactor

   ii. An **open system** made up of a 1,000 L raceway pond

   iii. A **split system**, consisting of the photobioreactor for growing and the raceway pond for lipid induction.

**Aim 2 Development of a cost-effective downstream process (harvesting and dewatering system of microalgae biomass)**

A. A study to check the feasibility of Flotation for marine microalgae (M8).

B. Method to determine the hydrophobicity of microalgae.

C. Comparison between Mechanical cell and Jameson cell.

D. Changes in collectors and hydrophobicity following the change in pH.

E. Use of chemical free flotation for microalgae.

**Aim 3 Application of optimised parameters for a large and cost-efficient microalgae production module**
Reference


1. Literature Review
A: Commercial Large-Scale Cultivation for Biofuels Feedstocks

Background

In green microalgae, the light-harvesting complex captures light energy as photons. These photons are used by photosystem II for catalytic oxidation of water, which produces protons, electrons, and molecular O$_2$ [1]. Low-potential electrons are then transferred through the photosynthetic electron transport chain and form NADPH by reducing ferredoxin. After water oxidation into the thylakoid lumen, proton release results in formation of an electrochemical gradient, which is used to drive ATP production via ATP synthase. NADPH and ATP formed are used as substrates for the Calvin–Benson cycle where inorganic CO$_2$ is fixed into 3-C molecules that are assimilated into the sugars, starch, lipids, or other molecules required for cellular growth [1].

When certain microalgae are grown under stressful conditions, they increasingly produce lipids/oils in the form of TAG’s (triacylglycerol), consisting of three long chain fatty acids attached to a glycerol backbone. These TAGs can then be transesterified with alcohol to form biodiesel (Fatty acid methyl ester (FAME)) [2]. Microalgae have numerous advantages over higher plants (crops) used for biodiesel production:

- Microalgae can be grown in salt or brackish water [3], thus not competing for valuable freshwater resources
- Microalgae can produce more oil (up to 80% of dry weight) when compared to oilseeds plants [2, 4].
- Microalgae can help in CO$_2$ fixation as they account for ~50% of global organic CO$_2$ fixation [4, 5].
- Microalgae can also provide other value-added commercially viable by-products (e.g. omega-3 fatty acids, carotenoids and animal feed).
- Depending on the climate, microalgae can be cultured in open ponds and photo-bioreactors throughout the year [2].
- Relatively less area is required for microalgae cultivation when compared to other plant crops [6].
- Microalgae can adapt to live in a variety of environments [7].
- Microalgae cultivation does not need to compete with arable land or biodiverse landscapes.

During mass culturing of microalgae there are two main phases; the first being a growth phase wherein maximum biomass is produced by keeping the culture in exponential phase by constantly supplying nutrients. The other phase is lipid induction, achieved by subjecting microalgae to stress. The most convenient and cost-effective stress method is nutrient starvation [8]. Based up on my experimental data, lipid induction might take up to two to three days depending on the weather conditions and species.

The main types of microalgae culturing systems are: - **Single cultivation systems** and **Split/Hybrid systems**. Single cultivation systems could be open-type, comprising of High Rate Production (HRP) ponds / raceways ponds or closed systems involving photo-bio reactors. In single cultivation systems, algae cultivation and lipid induction is carried out in a single production system; whereas in a split cultivation system, two different production systems are utilised for algae cultivation and lipid induction. The culturing method could be batch, semi continuous or continuous [9].

**Open pond production system**

Algae cultivation in open ponds has been in use since the 1950’s [10]. The main types of open systems are HRP and raceway ponds; both can be natural or artificial. Among these, commonly-used systems are closed loop oval shaped recirculating raceways [11, 12]. Mixing of the culture is done constantly using paddle wheels or compressed air in order to prevent sedimentation. Compared to closed photo-bioreactors, raceways are cheaper to build and operate [4, 13].

In raceway ponds, cooling is achieved by evaporation of water. Although this helps in maintaining temperature, water loss is significant [12] and could alter the salinity when seawater is used to culture marine microalgae. Since raceway ponds are open
systems, the loss of supplied CO₂ is significant and may result in lower biomass productivity [7] and insufficient mixing. Furthermore, open ponds are susceptible to contamination and pollution from other algae, protozoa or bacteria [11]. Since open ponds are only exposed to light from above, overall surface area becomes significantly smaller which may result in poor light penetration [14].

Closed production system (CPS)

Closed systems are flexible and allow growth conditions to be optimised according to the physiological and biological characteristics of microalgae. They also have a smaller area foot-print in terms of yield when compared to open systems [6, 15]. Closed photo-bioreactor systems (CPS) include tubular, flat plate and column photo-bioreactors. As these bioreactors are completely closed, the chances of contamination from foreign species are minimised and a single species can be grown consistently [12]. The microalgae culture is recirculated using a mechanical pump or airlift system. Compared to open systems, mixing is homogenised and maximum CO₂ diffusion can be achieved [16].

Tubular photo-bioreactors are made up of two sections, an airlift system and a transparent receiver. The airlift system allows the removal of O₂ from the system and addition of CO₂, as well as providing a means to harvesting and mixing the culture [17]. The transparent receiver provides a platform for the algae to grow. As they are made up of transparent material, the surface area of a bioreactor is much greater, thus increasing light penetration for higher biomass productivity. Furthermore, closed reactors prevent water evaporation, but their major drawbacks are:

a) Closed bioreactors are susceptible to overheating.

b) Formation of bio-fouling and dissolved oxygen accumulation.

c) Increased construction cost compared to open system with a high labour intensive maintenance.
Single cultivation system

In a single cultivation system, there is no separation between the growth and lipid induction phase, both of which occur one after the other and with an overlapping phase. Unfortunately, there is always the possibility that the microalgal culture enters the stationary growth phase as nutrients are depleted. Hence, microalgae cultivation could be carried out in a batch mode (once lipids are induced in the culture algae can be harvested and the system re-inoculated). However, this is labour intensive and thus increases operation costs. Furthermore, microalgal species with a slow growth rate may have a long lag phase take longer to reach the exponential phase, thereby increasing the duration of each cycle. To overcome these problems, a hybrid cultivation system will be tested as a part of this study.

Hybrid cultivation system

The hybrid cultivation system, also known as the split system, involves using two separate systems for growth and lipid induction [11]. During exponential growth, microalgae are cultivated in the photo-bioreactor, where contamination and growth parameters (e.g. mixing, pH and CO₂ diffusion) are easily controlled, resulting in higher biomass concentration. For the lipid induction phase, microalgae culture is transferred to open raceway ponds where lipids are induced through nutrient starvation. The transfer from closed to open system further increases lipid accumulation [4, 18] due to some additional environmental stress. Furthermore, the lack of nutrients in the open system and the short cultivation time decrease the chances of contamination [6]. Thus a hybrid production system helps to keep algae...
in the exponential phase for longer span of time and could minimise the labour and other cultivation cost.

A study carried out by Rodolfi et al. suggests that a hybrid system could give annual lipid production between 20 to 30 tonnes ha\(^{-1}\) depending upon the climatic conditions [4]. Huntley and Redalje used a split system for cultivation of *Haematococcus pluvialis*, and achieved an annual average oil production rate greater than 10 tonnes ha\(^{-1}\) per annum. They also demonstrated that, using higher lipid content species, it is possible to reach 76 tonnes ha\(^{-1}\) per annum [18].

Table 1: Comparison of various algae cultivating methods

<table>
<thead>
<tr>
<th>Factor</th>
<th>Open ponds</th>
<th>Photo-bioreactor</th>
<th>Split/hybrid system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space required</td>
<td>High</td>
<td>Moderate</td>
<td>High</td>
</tr>
<tr>
<td>Evaporation loss</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>CO(_2) spurring efficiency</td>
<td>Low</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Oxygen concentration</td>
<td>Low</td>
<td>High</td>
<td>High during growth</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low during stress</td>
</tr>
<tr>
<td>Shear</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Maintenance</td>
<td>Easy</td>
<td>Difficult</td>
<td>Moderate</td>
</tr>
<tr>
<td>Contamination risk</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Biomass quality</td>
<td>Variable</td>
<td>Reproducible</td>
<td>Reproducible</td>
</tr>
<tr>
<td>Mixing</td>
<td>Difficult</td>
<td>Easy</td>
<td>Moderate</td>
</tr>
<tr>
<td>Operation type</td>
<td>Batch</td>
<td>Batch</td>
<td>Continuous</td>
</tr>
<tr>
<td>Setup Cost</td>
<td>Low</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>Maintaining Continuous exponential phase</td>
<td>Difficult</td>
<td>Difficult</td>
<td>Easy</td>
</tr>
</tbody>
</table>

Thus, the species selection and production system design are two crucial criteria. Their ability to operate efficiently together is essential for successful microalgal production. Furthermore, in the past few years it has become evident that the use of freshwater for microalgae cultivation may result is scarcity of freshwater. Therefore, a special emphasis has been given in recent years to mass cultivation of algal species, grown in various saline waters ranging from brackish water to hyper-saline. *Tetraselmis* sp. (here and after termed as strain M8) is a local marine microalga collected from a coastal rock pool in South East Queensland [19]. Preliminary data collected from lab-scale work indicate that the preferred temperature range for this alga is similar to the average temperature of Brisbane. Therefore, M8 was used in this study for the comparison between three cultivation methods.

Selection of the appropriate cultivation system is a partial solution for the bigger problem, which is concentrating the cultivated microalgae for further downstream
processes. To date, there is no single superior method for harvesting microalgae which has high efficacy and low capital cost with minimum energy consumption.

Reference
Background

Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. However, microalgae harvesting/dewatering is a major obstruction to industrial-scale processing for biofuel production. The dilute nature of microalgae in cultivation creates high operational costs for harvesting, thus making microalgal fuel less economical. Within the last decade, significant advances have been made to develop new technologies for dewatering or harvesting of microalgae.

The in-depth literature about various dewatering techniques has been discussed in the review article: Critical analysis of current microalgae dewatering techniques. Published in Biofuels. From the review, flotation was identified to be a more cost-effective method for primary dewatering when compared to centrifugation, which is currently the most common method used for dewatering.

Froth flotation

Froth floatation is a highly versatile method for physically separating particles. Separation is based on differences in the ability of air bubbles to selectively adhere to specific particle surfaces [1]. It consists of three phases: water, solid particles and air bubble. Fine bubbles with diameters of about 1 mm are generated either by agitation combined with air injection or by bubbling air through porous media [2].

During the initial stages of collision, based on the deformation of bubbles, a thin liquid film referred to as wetting film is formed between the bubble and the particle [3]. Destabilisation of this film results in adhesion of hydrophobic particles to the air bubble, which is the basis for froth flotation and the most important mechanism in flotation [1, 4, 5]. Not all colliding particles attach to the bubbles; four forces affect particle-bubble attachment: Van der Waals force, electrostatic force, hydrodynamic interaction, hydrophobic interaction [5,6]. The most important force considered amongst them is hydrophobic interaction [5].
Hydrophobic interaction

_Laskowski and Kitchener_ were first to recognise the existence of hydrophobic interaction [5]. This interaction has to do with the property of bubbles to favour and attach to other hydrophobic particles. _Ducker et al_ found that the hydrophobic force is the primary force for attachment of hydrophobic particles to air bubble [5].

If particles and bubbles are oppositely charged, they tend to form bubble-particle aggregates due to electrostatic attraction. However, this attraction is so weak that the particles can get detached from the surface of bubbles easily (especially for large particles) [5]. Furthermore, if particles and bubbles have the same charge, it is unlikely that electrical forces are responsible for interaction. On the other hand, _Van der Waals forces_ are repulsive when considered with respect to bubble–particle interactions. Therefore, only when particles are sufficiently hydrophobic, the water films between the particles and bubbles get ruptured, and interaction takes place [8]. Thus if we consider a mixture of hydrophobic and hydrophilic particles (algae in this case) that are suspended in water and air is bubbled through the algae culture, only the hydrophobic algae will tend to attach to the air bubbles and float to the surface. Apart from the four forces, there are various external factors that affect flotation and particle-bubble interaction. These factors can be broadly classified into a three-cornered interactive system: chemical, equipment and operational component [2] (Figure 2).

![Figure 2: Three-cornered interactive system affecting flotation and particle (algae)-bubble interaction](image-url)
• Chemical components/reagents are needed in order to improve the flotation efficiency and can be classified into four major types; collectors, frothers, pH and modifiers [8].

• Equipment components are flotation cell design, agitation, air flow, cell bank configuration and cell bank control [8].

• Operation components are feed rate, particle size, pulp density and temperature [8].

Collectors

Collectors are organic chemical compounds used for flotation, consisting of a hydrophilic and a hydrophobic portion [2]. Depending upon their ability to dissociate, they can be of two types; ionising and non-ionising. Ionising collectors can further be divided into cationic (positively charged) or anionic (negatively charged) collectors [5,9] (Figure 3).

![Figure 3: Division of collectors](image)

A microalga’s surface can be made hydrophobic by adding collectors. When collector is added to the microalgal culture, the hydrophilic portion is adsorbed onto the microalgal surface while the hydrophobic portion projects out into the water. This renders the microalgae hydrophobic. When ionising collectors are used, they dissociate in the water, causing an ionic interaction between the microalgae and the collector. In the case of microalgae, mainly cationic collectors will be more effective as they possess a positive charge opposite to that of algae. This would aid in better bonding under alkaline conditions. Once microalgae have been rendered hydrophobic, they can attach to air bubbles and float to the surface. It is
hypothesised that as the hydrophobicity of algae would increase there will be an increase in overall microalgae recovery and it should continue to increase till the Critical Micelle Concentration of the collector has been reached. SDS, CTAB and Triton X-100 have been used by researchers for harvesting algae using froth flotation. These collectors have a range of pH at which they are most active [10,11]. Collector concentration has a significant effect on the removal of algae from water. Phoochinda et al. found that if the CTAB concentration was increased from 25 to 100 mg L⁻¹ there was gradual increase in the algae removal, but any further addition of CTAB resulted in a decrease of algae recovery [12].

**pH**

pH alters the action of the collector by either enhancing or reducing its adsorption on the surface of a particle. Particles may possess a different surface charge in acidic and alkaline pH. For instance, under acidic pH particles to be floated possess a positive charge, due to which they bind more efficiently with anionic collectors when compared to cationic collectors [10, 12]. When the pH is neutral or alkaline; particles bind efficiently to cationic collector, as particles are negatively charged. pH modifiers play a critical role in flotation of particles by affecting particle surface characteristics [9]. The common modifiers used to control pH are sodium hydroxide (NaOH), lime, sodium carbonate, ammonia (NH₃), hydrochloric acid (HCl) and sulphuric acid (H₂SO₄) [12].

Optimum collector dosage and pH help in bringing the particle charge close to zero and produce particles with a more hydrophobic property so that particle-bubble interaction can take place [5]. Bubble size and froth stability are also very important for high rate flotation.

**Bubble size**

Bubble size has strong influence on the rate of particle removal [13]. Smaller air bubbles rise slowly, increasing the retention time and collision hydrodynamics, thus the probability of particle-bubble collision increases. As a result, collision rate and attachment efficiency also increase. Small bubbles are desirable for fine particle flotation, however, if the bubble size is too small, the buoyancy force to lift the attached particles to the surface may not be sufficient [5,13].
Bubble coalescence

Bubble coalescence incorporates the thinning of liquid film, followed by the final rupture of the thin liquid film between two bubbles [2]. The use of frothers or a high salinity have prevented the coalescence of air bubble, even high salinity prevents coalescence [14].

Frother

Frother is chemical compound that help to stabilise the air bubble and keep them well dispersed in the algal culture [13]. The froth that is formed can be removed before bubbles burst. One of the most widely used frother is MIBC (methyl isobutyl carbonyl) [2].

Ionic strength (salinity)

Salts have been found to lower the percentages of bubble coalescence occurring as well as reduce the size of the bubbles generated [14]. Most of the studies carried out on the effect of ionic strength on flotation in mineral processing field indicates that sea water could act as natural frother [2, 14]. Theoretically with the presence of salts, there is a formation of an electrical double layer (EDL) around the surface of a particle. As the zeta potential is measured at the first layer; the presence of EDL results in a lower surface charge, thus increasing electrostatic interaction and increasing particle-bubble interaction. However, all available information on fresh water microalgae suggests that flotation is significantly affected by ionic strength. It is suggested that lower ionic strength solutions have better removal due to the decrease in zeta potential and weakened electrostatic interactions [10, 11] (Figures 4 & 5) [11, 15].
Figure 4: Effects of ionic strength on the removal of *Scenedesmus quadricauda* when SDS was used as the collector. [10]

Figure 5: Effects of ionic strength on the removal of *Chlorella* sp. when SDS was used as the collector. [Chitosan] = 10 mg/L, [SDS] = 20 mg/L, pH 8.0 ± 0.1, I = 0.05 M NaNO3, initial cell concentration (C0) = $6.8 \times 10^5$ cells/mL, air flow rate = 114 mL/min. [11]

**Modifiers**

Modifiers are chemicals that could influence the collector's attachment to particle surfaces. They act by increasing or preventing a collector from adsorbing onto a particle. For example, chitosan is a positively charged compound which binds on the
surface of algae and gives them positive charge. When added to a microalgal culture, flotation efficiency with anionic collector increases and the efficiency decrease for cationic collectors [10,11].

A study by Liu et al [10] shows that when SDS was used as collector at pH 8, addition of chitosan improved algae recovery to 90%. Under similar conditions when CTAB was used as collector, efficiency decreased by 50% (Table 2).

<table>
<thead>
<tr>
<th>CTAB 20 mg/L</th>
<th>SDS 20 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>chitosan</td>
<td>chitosan</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Table 2: Algae recovery with Chitosan (surface modifier), using different collectors

Generally, microalgal cells carry a negative charge that prevents aggregation of cells in suspension [16]. The reason for poor recovery with SDS at neutral or alkaline pH could be due to the similar charge of SDS and algae. On addition of chitosan; the algae surface charge becomes positive and SDS could bind efficiently at neutral or alkaline pH.

Other factors that affect the flotation process are airflow rate, conditioning time and agitation [8]. Based on previous studies on Scenedesmus quadricauda & Chlorella sp., it was found that cationic CTAB collectors were the most effective compared to anionic SDS and nonionic Triton X-100 collectors (optimum pH range of 5.0 to 8.0). However, upon the addition of chitosan (positively-charged polymer compounds that act as coagulants and activators), the performance of SDS was significantly improved. It has also been reported that the addition of salts to algae culture (increasing the ionic strength of the culture) reduces the algae recovery efficiency. It is assumed that the reason behind the reduced efficiency is the incapability of collectors to work at high ionic strengths.

Furthermore, when comparing Figures 4 & 5 under similar conditions, Chlorella sp. had lower removal rate when compared to Scenedesmus quadricauda [10, 11]. This indicates that the high ionic strength is not necessarily the only reason behind decreased microalgae recovery. There are another factor that needs to be considered is the surface chemistry of the microalgae and one of them is hydrophobicity. The role of surface hydrophobicity has not been investigated much.
This research project aims to identify the role of surface chemistry in different algae and its effects on harvesting. Studies will also focus on measuring their hydrophobicity as well as develop methods to increase the hydrophobicity of algae.

**Key definitions**

Recovery: The microalgae in the original feed that is recovered in the concentrate.

Concentrate: The froth collected from the flotation cell which contains all the recovered microalgae in it.

Tailings: Tailings are the materials left over after the process of separating the hydrophobic microalgae from the leftover non-hydrophobic microalgae

**References**


CRITICAL ANALYSIS OF CURRENT MICROALGAE DEWATERING TECHNIQUES

Sourabh Garg ††, Kalpesh K Sharma ††, Yan Li ‡‡, Ali Malekizadeh ‡ & Peer M Schenk *†
1 Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane QLD 4072, Australia.
2 School of Tropical and Marine Sciences, James Cook University, Douglas, QLD 4811, Australia

* Author for correspondence †These authors contributed equally

Abstract

Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. However, microalgae harvesting/dewatering is a major obstruction to industrial-scale processing for biofuel production. The dilute nature of microalgae in cultivation creates high operational costs for harvesting, thus making microalgal fuel less economical. Within the last decade, significant advances have been made to develop new technologies for dewatering or harvesting of microalgae. The choice of which harvesting technique to apply depends on the microalgae cell size and the desired product. Microalgae dewatering processes can broadly be classified as primary and secondary dewatering. This article provides an overview of current dewatering techniques along with a critical analysis of costs and efficiencies, and provides recommendations towards cost-effective dewatering.

Introduction

Microalgae have a robust photosynthetic capability for fixing CO₂ and converting solar energy into chemical energy. Moreover, they do not need to compete with arable land and freshwater, and have been considered as one of the most promising feedstocks for biofuels [1,2]. Microalgae are typically 2–50 µm in size with a negative charge on the cell surface [3–5], but some microalgae, under certain conditions, have a larger cell size. In most cases they are motile (i.e., swimming or gliding), such as dinoflagellates or raphid diatoms, and form stable suspensions. Unfortunately, microalgal biomass is fairly dilute in cultures (up to 0.3–0.5 g dry biomass/l), resulting in difficulties in harvesting and dewatering algae cost effectively [6]. Microalgae harvesting can typically make up to 20–30% of the total
biomass production cost [7–9]. This makes the harvesting process a major bottleneck, hindering the development of the microalgae industry. To date, there are a multitude of techniques being used for microalgae dewatering, but with low economic feasibility. Based on their large biodiversity, microalgae harvesting processes are to a large extent species specific [10,11]. They are also closely linked to cell density and cultivation conditions [12].

The production of biofuel, such as biodiesel, from microalgae is a multistep process involving cultivation, biomass harvest, lipid extraction and oil conversion. Compared with the other processes, harvesting is arguably still the most critical and challenging stage in microalgae biomass production [4,8,12–15]. When considering commercial-scale processes for dewatering and recovering algal biomass for further downstream processes, a traditional harvesting method may involve up to two steps; the first is known as primary harvesting/bulk harvesting, and the second is known as secondary dewatering/thickening (Figure 1) [8–10,16]. During the primary harvesting process, the microalgae mass ratio to water volume is increased [17]. This step aims to achieve a concentration containing 2–7% total solid matter, from the initial biomass concentration [16]. Secondary dewatering concentrates the biomass up to 15–25%, which when followed by drying, aims to further concentrate the slurry, increasing the total solid matter up to 90–95%. This step is generally a more energy-intensive step than primary harvesting. Several techniques for dewatering of microalgae cultures have been developed [16].

This article attempts to provide an overview of these techniques to estimate their efficiencies, and then classify these techniques based on their properties. It also highlights the need for developing hybrid technology. It is desired to optimize microalgae dewatering processes by combining the strengths of several different harvesting techniques.
Primary harvesting

Primary harvesting methods reviewed here include flocculation, flotation, sedimentation and electroflocculation (Figure 1) [1,6,18].

Flocculation

Flocculation is often performed as a pre-treatment to increase the particle size before using another method (Table 1). Hence, flocculation is commonly used before secondary dewatering processes to facilitate further steps such as centrifugation or filtration [4,10,19]. In some cases negative charges of microalgae cells inhibit aggregation; therefore, cationic flocculants, cationic polymers and metal salts (e.g., ferric chloride, alum, aluminum sulfate and ferric sulfate) are used to neutralize charges and facilitate aggregation [4,13,16,19–22]. The efficiency of electrolytes to induce coagulation is measured by the critical coagulation concentration, or the concentration required to cause rapid coagulation. Coagulation efficiency of metal ions increases with increasing ionic charge. Multivalent metal salts such as alum have been widely used to flocculate algal biomass in wastewater treatment processes [23,24]. Alum is an effective flocculant for freshwater species such as Scenedesmus and Chlorella [25]; however, for maximizing the economic value
derived from the feedstock, there is a need to produce various co-products such as pigments, protein, omega-3 fatty acids and animal feed along with biofuel production \[26\]. Hence, flocculation by metal salts may be unacceptable if biomass is to be used in certain aquaculture applications or to be used as food or feed. Polyferric sulphates are reported to be a better flocculant compared with the more traditional nonpolymerized metal salt flocculants, as shown by Jiang et al.\[27\]. Prepolymerized metal salts are effective over a wider pH range than nonpolymerized salts \[27\]. Moreover, flocculation was carried out by adjustment of pH using sodium hydroxide and addition of the nonanionic polymer Magnafloc LT-25 to a final concentration of 0.5 mg l\(^{-1}\) by Knuckey et al. \[28\].

Ultrasound has also been used to induce aggregation in microalgae \[29\]. Microbial flocculation under nutrient-depletion stress has been investigated by Lee et al. \[30\]. Flocculation occurs naturally in some microalgae; for example, by high light, nitrogen stress, and changes in pH, salinity or the level of dissolved oxygen \[22\]. This typically leads to flocculation and settling, and probably presents a protective survival mechanism for algae in their natural environment.

Electrolytes and synthetic polymers are typically added to coagulate (neutralize charge) and flocculate the cells, respectively \[31\]. Smith and Davis recently investigated autoflocculation using magnesium-based flocculants naturally available in brackish water \[32\]. Moreover, magnesium-based flocculants can be obtained from wastewater treatment plants. A recent study carried out by Taylor et al. on *Nannochloropsis oculata* observed that artificially treating the algae with algal extracts does not only effectively flocculate microalgae, but also increase overall lipid content \[33\]. Interrupting CO\(_2\) to algae culture may also cause auto flocculation \[34,35\]. However, auto flocculation may not be as reliable as chemical flocculation \[22\]. Electrocoagulation flocculation (ECF) has been evaluated as a method for harvesting a freshwater (*Chlorella vulgaris*) and a marine (*Phaeodactylum tricornutum*) microalgal species by Vandamme et al. \[36\]. In this study, ECF was shown to be more efficient using an aluminium anode than using an iron anode. Moreover, the efficiency of the ECF process could be substantially improved by reducing the initial pH and by increasing the turbulence in the microalgal suspension. In another study conducted by Xu et al., a rapid and efficient
Table 1 Examples of various flocculation studies that have been used to harvest microalgae

<table>
<thead>
<tr>
<th>ALGAE</th>
<th>FLOCULANT</th>
<th>SOURCE</th>
<th>pH</th>
<th>DOSAGE</th>
<th>RESULT</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tetraselmis suecica</em></td>
<td>Praestol</td>
<td>Acrylamide (Non-Biological)</td>
<td>8.2</td>
<td>1 mg L$^{-1}$</td>
<td>70% in 30 mins</td>
<td>[47]</td>
</tr>
<tr>
<td><em>Spirulina platensis</em></td>
<td>Praestol</td>
<td>Acrylamide (Non-Biological)</td>
<td>9.4</td>
<td>1 mg L$^{-1}$</td>
<td>70% in 30 mins</td>
<td>[47]</td>
</tr>
<tr>
<td><em>Rhodopseudomonas palustris</em></td>
<td>Praestol</td>
<td>Acrylamide (Non-Biological)</td>
<td>6.8</td>
<td>1 mg L$^{-1}$</td>
<td>86% in 30 mins</td>
<td>[47]</td>
</tr>
<tr>
<td><em>Chaetoceros calcitrans</em></td>
<td>Magnafloc ®LT25</td>
<td>Polyacrylamide (Non-Biological)</td>
<td>10.2</td>
<td>&gt;1 mg L$^{-1}$</td>
<td>93% in 4 hours</td>
<td>[48]</td>
</tr>
<tr>
<td><em>Chaetoceros calcitrans</em></td>
<td>Chitosan</td>
<td>Inorganic polymer (Biological)</td>
<td>8.0</td>
<td>20 mg L$^{-1}$</td>
<td>83% in 4 hours</td>
<td>[48]</td>
</tr>
<tr>
<td><em>Chlorella minutissima</em></td>
<td>Alumminium Chloride</td>
<td>Inorganic salt (Non-Biological)</td>
<td>-</td>
<td>0.5 mg L$^{-1}$</td>
<td>90% in 5 hours</td>
<td>[49]</td>
</tr>
<tr>
<td><em>Scenedesmus</em></td>
<td>Greenfloc 120</td>
<td>Cationic starch</td>
<td>-</td>
<td>&gt;10 mg L$^{-1}$</td>
<td>&lt;90% in 30 + 30 mins</td>
<td>[45]</td>
</tr>
<tr>
<td><em>Phaeodactyllum tricornutum</em></td>
<td>Sodium hydroxide</td>
<td>Alkaline agent</td>
<td>9.8 – 10.61</td>
<td>-</td>
<td>90 – 97% in 1 hour</td>
<td>[50]</td>
</tr>
<tr>
<td><em>Phaeodactyllum tricornutum</em></td>
<td>Chitosan</td>
<td>Inorganic polymer (Biological)</td>
<td>9.9</td>
<td>20 mg L$^{-1}$</td>
<td>90% in 30 mins</td>
<td>[50]</td>
</tr>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>Magnesium + Sodium Hydroxide</td>
<td>-</td>
<td>10.5</td>
<td>0.15 mM</td>
<td>&lt;90% in 30 + 30 mins</td>
<td>[51]</td>
</tr>
</tbody>
</table>
electroflocculation method integrated with dispersed air flotation was developed for harvesting Botryococcus braunii with a recovery of 98.6% within 14 min [37].

When considering downstream processes to produce bioproducts from algae, the use of metal salts for coagulation and flocculation poses many challenges. In wastewater sludge treatment, aluminum and sulfate have been shown to affect the specific methanogenic activity of methanogenic and acetogenic bacteria, and reduce their anaerobic digestion ability [38]. A similar problem may be faced when using algal biomass for anaerobic digestion. Land application of aluminum-treated sludge can increase heavy metal uptake and cause phosphorus deficiency in plants [39].

Natural polymers that do not raise environmental concerns may also be used as flocculants, although these are less studied. One of the most widely used and studied natural polymers for flocculation is chitosan (at a pH of ~7), which is typically derived from crab shell. Divakaran et al. reported successful flocculation and settling of algae by adding chitosan [40], which is considered an environmentally friendly option that has also been used in various other studies [41–43]. Other nonconventional flocculants such as Moringa oleifera seed flour has been used by Teixeira et al. as another nontoxic microalgae flocculant [44]. Cationic starch is also mentioned as another potential effective flocculant for freshwater microalgae by Vandamme et al. [45].

**Gravity-assisted sedimentation**

This process is commonly used in wastewater treatment. However, this process is also appropriate for microalgae larger than 70 µm in size [16,46], but is typically fairly slow due to the low specific gravity of algal cells [4].

**Flotation**

In this process, microalgal cells are trapped on microair bubbles and float to the surface [16]. Efficient flotation relies on successful collision and attachment of bubbles and particles, and works best when algal cells are hydrophobic [3,47].

Dissolved air flotation (DAF) has been successfully used in water treatment plants and is also widely used for microalgae harvesting (Table 2) [48,49]. It involves the
release of pressurized water (saturated with air) into the tank containing microalgae. Due to the difference of pressure, many fine bubbles form, carrying algal cells as a froth, which can be skimmed off. The effectiveness of this process depends on air bubble size, solubility and the pressure difference of air, the hydraulic retention time, and the floated particle size [50]. Before algae can be removed using DAF they need to be flocculated. The flocculation increases the efficiency of removal. A study carried out by Edzwald found DAF to be more effective than sedimentation [50]. Suspended air flotation is an alternative method that could potentially harvest microalgae with a lower air:solids ratio, lower energy requirements and higher loading rates compared with DAF [51].

In dispersed air flotation (or foam flotation), algal cells are floated in a mechanical cell with a high-speed agitator through which a constant stream of air is passed [3]. Fine bubbles of approximately 1 mm diameter are generated by either ‘agitation combined with air injection’ or ‘bubbling air through porous media’ [52]. Hydrophobic interaction plays an important role for attachment particles, such as microalgae, to the bubbles [3]. Bubbles then rise to the surface and constantly accumulate as foam as a result of solid–liquid separation [3]. Foam fractionation is considered as an alternative to the use of expensive centrifugation for microalgae harvesting [53].

**Secondary dewatering**

In secondary dewatering or thickening, the algae slurry is concentrated approximately 10–30-times, and consequently the water content of the produced algae paste can be as low as 20–25% (Figure 1) [13]. Energy-intensive processes such as centrifugation and ultrasonic aggregation are commonly used at this stage [16]. This step requires more energy input than primary dewatering, and therefore needs more capital and operating costs [46].

**Centrifugation**

Centrifugation is the ideal method for rapid harvesting of algae containing high-value products. Generally centrifuges can be of various types and sizes depending on the uses. A disc stack centrifuge consists of a relatively shallow cylindrical bowl containing a number (stack) of closely spaced metal cones (discs) that rotate, and it is mostly used in commercial plants for high-value algal products and in algal biofuel
Table 2: Examples of various flotation studies that have been used to harvest microalgae

<table>
<thead>
<tr>
<th>ALGAE</th>
<th>SURFACTANT</th>
<th>SURFACANT TYPE</th>
<th>pH</th>
<th>DOSAGE</th>
<th>RESULT</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Scenedesmus quadricauda</em></td>
<td>SDS + Chitosan</td>
<td>Anionic surfactant</td>
<td>8.0 – 5.0</td>
<td>20 + 10 mg L⁻¹</td>
<td>95% in 20 mins</td>
<td>[82]</td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>8.0</td>
<td>40 mg L⁻¹</td>
<td>86% in 20 mins</td>
<td>[83]</td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>SDS + Chitosan</td>
<td>Anionic surfactant</td>
<td>8.0 – 5.0</td>
<td>20 + 10 mg L⁻¹</td>
<td>85-90% in 20 mins</td>
<td>[83]</td>
</tr>
<tr>
<td><em>Scenedesmus quadricauda</em></td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>7.8</td>
<td>100 mg L⁻¹</td>
<td>&gt;90% in 20 mins</td>
<td>[84]</td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>9.5</td>
<td>1-3 mg L⁻¹</td>
<td>95-99% in 11 mins</td>
<td>[3]</td>
</tr>
<tr>
<td><em>Chlorella sp.</em></td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>9.5</td>
<td>10 mg L⁻¹</td>
<td>45 mins</td>
<td>[85]</td>
</tr>
<tr>
<td><em>Dunaliella salina</em></td>
<td>Aluminium sulphate</td>
<td>Inorganic metallic coagulants</td>
<td>5</td>
<td>150 mg L⁻¹</td>
<td>95% in 30 mins</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td>Ferric sulphate</td>
<td></td>
<td>5</td>
<td>150 mg L⁻¹</td>
<td>98% in 30 mins</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric Chloride</td>
<td></td>
<td>5</td>
<td>75 mg L⁻¹</td>
<td>98.7% in 30 mins</td>
<td></td>
</tr>
</tbody>
</table>
pilot plants. Decanter centrifuges have been found to be as effective as solid-bowl centrifuges for separating microalgae, but the energy consumption of decanter centrifuge is higher than that of disc-bowl centrifuges at 8 kWh m$^{-3}$ [8]. A hydrocyclone is a relatively low-energy (0.3 kWh m$^{-3}$) particle-sorting device compared with other centrifuge methods, but on the other hand it was reported to be an unreliable means of concentrating microalgae as only a maximum concentration factor of 4 could be achieved [8]. Spiral plate centrifuges are considered a relatively new generation of centrifuges, manufactured by Evodus. The suspension flows outwards in thin films over vertical plates with the solid sediment or microalgae being forced by centrifugal force to collect on the outer bottom edge of the vanes. Table 3 provides more analyses and details about the harvesting of 10,000 l of Chlorella sp. with an Evodos centrifuge. More detailed studies on centrifuge harvesting have been carried out by Molina Grima et al. [8]. However, centrifugation is energy intensive, not easily scalable and requires high maintenance due to fast-moving mechanical parts [8,16,22]. Therefore, centrifugation has high capital and operating costs, and is considered too expensive for low-value products such as biofuel [8,54]. Furthermore, high speed spinning can disrupt algae cells [19,55].

**Filtration**

Filtration methods such as microstrainers, vibrating screen filters, and micro- and ultra-filtration have been widely studied, and have proven to be efficient (Table 3) [56–58]. One of the major disadvantages of these techniques is the high capital and operating costs to avoid filter blinding and disruptive pressure changes (high pressure or vacuum). Membrane filtration and ultrafiltration are costly for large-scale operations due to high operating costs for membrane replacement, clogging and pumping [4,8,10,13,16,22,46,57]. Although the filtration process may be considered slower than centrifugation for some applications [10], it is still a simpler and lower cost alternative when compared with centrifugation, if implemented properly. Fast formation of thick filter cake, which dramatically decreases flow rate, is another disadvantage of conventional filtration processes[22].

Cross-flow filtration (tangential flow filtration) has been shown to solve these problems as the filter cake is washed away during the cross-flow filtration process,
which increases the operation time of the filtration system [59]. However, this technology is still very expensive for low-value products and is not easily scalable. In addition, most studies consider the conventional filtration process as unsuitable for harvesting of small microalgae (smaller than 30 µm) [4,10,13,16,22,60].

Table 3 Examples of various Flirtation studies that have been used to harvest microalgae

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of filtration</th>
<th>Effective</th>
<th>Reff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coelastrum sp</td>
<td>Non-Precoated vacuum drum filter</td>
<td>18% TSS</td>
<td>[6]</td>
</tr>
<tr>
<td>Coelastrum sp, Scenedesmus sp</td>
<td>Potato starch vacuum drum filter</td>
<td>37% TSS</td>
<td>[6]</td>
</tr>
<tr>
<td>Coelastrum sp,</td>
<td>Belt filter</td>
<td>9.5% TSS</td>
<td>[6]</td>
</tr>
<tr>
<td>Chlorella and Cyclotella</td>
<td>Micro filtration</td>
<td>-</td>
<td>[50]</td>
</tr>
<tr>
<td>Scenedesmus quadricula</td>
<td>ultra filtration membranes</td>
<td>-</td>
<td>[87]</td>
</tr>
<tr>
<td>Spirulina sp</td>
<td>ultra filtration membranes</td>
<td>20%TSS</td>
<td>[88]</td>
</tr>
<tr>
<td>Spirulina Micractinium</td>
<td>rotary vacuum filter</td>
<td>1-2% TSS</td>
<td>[89]</td>
</tr>
<tr>
<td>Spirulina</td>
<td>Belt filters</td>
<td>18% TSS</td>
<td>[8, 90]</td>
</tr>
<tr>
<td>Haslea ostrearia, Skeletonema costatum</td>
<td>Cross-flow microfiltration and ultra-filtration</td>
<td>-</td>
<td>[91]</td>
</tr>
</tbody>
</table>

Drying

The water content of algal paste after secondary dewatering should not exceed 50% before oil extraction [12]. Because the cost of thermal drying is high (even higher than mechanical drying), a harvesting method with a high solid content is preferable before drying [10]. Common methods for drying microalgae after secondary dewatering are: spray drying, drum drying, freeze drying and sun drying [10]. Spray drying is considered too expensive for low-value products such as biofuel [10]. The influence of short-term storage and spray and freeze drying of fresh microalgal paste on the stability of lipids and carotenoids of P. tricornutum was investigated by Ryckebosch et al. [61]. Solar drying is considered the most economical drying process; however, it requires large land areas for large-scale operations [12,62].
Techno-economic assessment

Using the information from previously completed studies and specifications provided by companies that supply the equipment and chemicals, a theoretical calculation was carried out to determine the technoeconomic feasibility of overall biomass recovery in a one-step as well as a two-step method. For the costing purpose, harvesting of 10,000 l *Chlorella* sp. culture was considered (Table 4). Assessments were independently developed in accordance with Australian conditions and, where possible, were compared to equivalent costing from previous economic analyses of microalgal biofuel systems.

Table 4 compares some of the traditionally used harvesting methods in microalgal bioprocessing. From the table it can be summarized that due to its high energy consumption, single-step centrifugation is the most expensive method when compared with other techniques.

Flotation appears to be the most cost-effective method for primary dewatering; however, if used with centrifugation, the overall setup costs will increase and would result in higher capital costs. On the other hand, for flotation, if used in conjunction with filtration, the overall process may become more feasible but there is still room for improvement. Moreover, the cetyl trimethyl ammonium bromide chemical used for flotation is not only toxic to the environment but also makes the biomass unfit for human and animal consumption. Flocculation coupled with filtration may be more cost effective, but chitosan used for flocculation is biodegradable, as it is derived from a biological source (crustacean). However, large-scale use of chitosan may not be possible as it is expensive, as well as this putting pressure on crustacean populations. Commercial chitosan is derived from the shells of shrimp and other sea crustaceans, including *Pandalus borealis*. Hence, some harvesting techniques are more feasible than others when considering costs only, but some of these may not be environmentally friendly. Thus, there is a need to optimize current methods or to develop improved methods that are not only cost effective, but also environmentally friendly.
Table 3 Cost of harvesting 10,000 l of Chlorella sp. with different harvesting techniques.

<table>
<thead>
<tr>
<th></th>
<th>Single step</th>
<th>Primary</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Centrifugation</td>
<td>Sedimentation</td>
<td>Flotation (1)</td>
</tr>
<tr>
<td><strong>Total Energy Consumed</strong></td>
<td>55 kWh/10 m³*</td>
<td>-</td>
<td>7.4-8.4 kWh/10 m³*</td>
</tr>
<tr>
<td><strong>Energy Cost (AUD)$</strong></td>
<td>$12.10*</td>
<td>-</td>
<td>$1.62 – $1.84*</td>
</tr>
<tr>
<td><strong>Dosage required</strong></td>
<td>-</td>
<td>100 g @ 10 mg L⁻¹ [1]</td>
<td>30 g @ 3 mg L⁻¹ [3]</td>
</tr>
<tr>
<td><strong>Chemical Cost (AUD)</strong></td>
<td>-</td>
<td>$2.50 (Chitosan @ $25/kg)</td>
<td>$0.24 (CTAB @ $8/kg)</td>
</tr>
<tr>
<td><strong>pH adjustment Dosage</strong></td>
<td>-</td>
<td>1.5 to 2 L acetic acid~</td>
<td>-</td>
</tr>
<tr>
<td><strong>pH Adjustment Cost</strong></td>
<td>-</td>
<td>$1.20 – $1.60 @ $800/ton</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total Cost (AUD)</strong></td>
<td>$12.10</td>
<td>$3.70 - 4.10</td>
<td>$1.86 – $2.08</td>
</tr>
</tbody>
</table>

$ Australian Dollar (= approx. US $1.04)

*Electricity prices were calculated based on $0.22 per kWh

† An Evodos centrifuge was used for this study [3]

+ Flotation cell considered is Jameson cell and energy consumption was determined using various published studies and our own unpublished data.

† Flotation cell considered is Column flotation cell and energy consumption was determined using work done by Coward et al.

~ The volume was estimated by doing an experiment with 1 L of algae culture and mathematical calculation.
Classification of current harvesting processes

Current harvesting methods mentioned above can be divided into chemically based, mechanically based and biologically based (Figure 2). Various combinations or sequences of these methods can be used for cost-effective harvesting. Currently, biologically based methods are being investigated as a cost-reducing and environmentally friendly means of harvesting [63]. In any case, it needs to be checked if any desirable valuable compounds are lost during the process. To develop a cost-effective harvesting technique, apart from the costs, one has to consider the following three main aspects: species-specific requirements of microalgae that need to be harvested; recovery/yield of desired product; and environmental impact.

Chemically based methods can be termed as a harvesting method that involves the addition of chemicals to the microalgae culture to induce flocculation, which is used in various solid–liquid separation processes as a pre-treatment stage [64]. The chemical reactions are highly sensitive to pH, and high doses of flocculants are required to produce large amounts of sludge, which may leave a residue in the treated effluent. Although cost
effective, a major disadvantage could be the presence of harmful salts and chemicals in the extracted biomass, which can possibly pose health and environmental risks. For example, use of aluminium oxide to flocculate microalgae can lead to accumulation of aluminium salt precipitates in the biomass.

Mechanical harvesting, as the name suggests, is the method that involves the use of a mechanical machine to harvest microalgae, which generally includes centrifugation, filtration and flotation. Molina Grima et al. concluded that centrifugation is a preferred method for harvesting of microalgal biomass, especially for producing extended shelf-life concentrates for aquaculture, pharmaceuticals and other high-value products such as omega-3 [8]. However, Knuckey et al. state that exposure of microalgae cells to high gravitational and shear forces can damage the cell structure [28]. In addition, processing a large amount of culture using centrifugation is time consuming and increases the overall costs of microalgal biomass production (Table 4). Filtration and gravitational sedimentation are widely applied in wastewater treatment facilities to harvest relatively large (>70 µm) microalgae such as Coelastrum and Spirulina. However, they cannot be used to harvest algae species approaching bacterial dimensions (<30 µm) such as Scenedesmus, Dunaliella and Chlorella, which can rapidly and easily blind the filter [16]. This may result in higher operating costs and frequent replacement of filters. In summary, most technologies including chemical and mechanical methods greatly increase operational costs for algal production and are only economically feasible for production of high-value products [65].

Biological harvesting is the method in which bioproducts or other microorganisms are used for the harvesting of microalgae. When cultivating microalgae, some cultures tend to aggregate and grow as fluffy pellets, or tightly packed, compact or dense granules. These fluffy pellets are caused by filamentous microorganisms, including some species of molds and bacteria [66–68], and may assist in trapping additional microalgal cells, one of the major advantages of cell pelletization [7,63,68]. Fungal cell growth can be induced by changing operational conditions during cell cultivation, rather than using CaCO₃ powder or other nuclei to induce the fungal pelletization [66], which are costly and cause solid waste disposal issues. A preliminary study was recently conducted by Zhou et al. to inoculate filamentous fungal spores when culturing mixotrophic green algae, C. vulgaris, with the result that pellets clearly formed within 2 days of culture [68]. Microalgal cells, aggregated together with fungal cells, were immobilized in the pellets [67]. Bioflocculation using flocculating microalgae has also been investigated by others [63,68]. The advantage of
this method is that neither addition of chemical flocculants is required nor the cultivation conditions have to be changed. This method is as simple and effective as chemical flocculation; however, it is potentially more sustainable and cost effective. No additional costs are involved for pretreatment of the biomass before oil extraction and for the medium before it can be reused\cite{27}. An interesting method is the use of zooplankton to harvest microalgae \cite{69}. Biological harvesting could be a cost-effective method to harvest microalgae, but it is time consuming and has limitations in large-scale cultivation, as enough bioproduct must be co-produced. In addition, chances of cross-contamination are very high.

Based on our analyses conducted on microalgae harvesting technologies (Table 4), it is evident that harvesting techniques should not only be cost effective and rapid, but also have to be environmentally safe and easily scalable for a microalgae-based biorefinery industry. Thus, there is a need to think outside the box and develop new hybrid methods that may combine the best aspects of several techniques (Figure 2).

**Biochemically based methods**

As described above, flocculation assisted by chitosan (biologically derived) has been used in many studies on different microalgae and has proven to be very promising \cite{41,70,71}. Another example of biologically derived flocculation is the use of *M. oleifera* seeds, which have also been used for water treatment due to their high flocculation potential, low cost and low toxicity. Recently, Teixeira et al. demonstrated *M. oleifera* as a successful flocculating agent for *C. vulgaris* \cite{44}. In addition, a range of new bioflocculants are proposed to address the cost and environmental concerns for current flocculation methods \cite{14}. Microalgae flocculation was also achieved by using naturally available ions in brackish water, and a variety of precipitating ions, including Mg$^{2+}$, Ca$^{2+}$ and CO$_3$ $^{2-}$, can lead to autoflocculation of microalgae \cite{32}. A combination of bioflocculants together with a low dose of chemicals may lead to the best flocculation outcome.

**Emerging technologies**

When considering chemical, mechanical and biological harvesting methods, each method has its advantages and disadvantages. Biomechanical and chemical–mechanical methods for flocculation are less explored when compared with other methods. Developing hybrid techniques, which make use of all three harvesting categories, may be a viable option that is worth exploring.
The conceptual photobioreactor shown by Chen et al. has the potential to be developed into a commercially viable microalgae cultivation system with zero electricity consumption [56]. This was made possible by combining sunlight and multi-LED light sources with solar panels and a wind power generator. Similarly, when considering harvesting, electricity cost is the key factor that makes the process costly, but renewable energy sources such as solar and wind can be used to generate green electricity [72]. The main disadvantage of these systems is the high construction costs.

Another option to reduce the cost of harvesting could be by combining two or more stages of microalgal biodiesel production with a harvesting method into one step; for example, as done in the study carried out by Tayloret al. [33]. By doing so, not only can the cost be reduced, but also the overall time required for a full production cycle. For example, developing a process that can help in rapid induction of lipids as well as flocculation could accelerate the harvesting process. Similarly, a method was developed by Hejazi et al. for milking β-carotene from Dunaliella salina in a two-phase bioreactor [73]. In this technique, cells were first grown under normal growth conditions and then stressed by excess light to produce larger amounts of β-carotene, and later a biocompatible organic phase was added and the β-carotene was extracted selectively via continuous recirculation of a biocompatible organic solvent through the aqueous phase containing the cells. Because the cells continue to produce β-carotene, the extracted product was continuously replaced by newly produced molecules. Therefore, the cells are continuously reused and do not need to be grown again. Thus, in contrast to existing commercial processes, this method does not require harvesting, concentrating and disruption of cells for extraction of the desired product [73,74].

Matrix-attached algae culture systems have been developed for growing microalgae on the surface of polystyrene foam to simplify the cell harvest [7,75]. These methods are innovative and will decrease the harvesting costs to some extent if developed successfully, but require heavy investments on equipment and chemical supplies with various combinations or sequences of these methods. Xu et al. developed a simple and rapid in situ magnetic harvesting method by using Fe$_3$O$_4$ nanoparticles on B. braunii and Chlorella ellipsoidea. Magnetic particles were added to the microalgal culture broth and then separated by an external magnetic field [76]. Recently a genetically modified approach has also been used for harvesting microalgae of genera Chlamydomonas, Dunaliella, Scenedesmus and Hematococcus sp. [76].
Conclusion

When considering the research carried out in the field of harvesting microalgae over the past few decades, much progress has been made. Researchers have optimized various techniques; machines have become more energy efficient. There is a need to optimize current methods or to develop improved techniques that are not only cost effective, but also environmentally friendly. Moreover, there is a need to develop hybrid harvesting technology that can use the best of all current harvesting methods. The costing calculation in this review suggests that flotation for primary dewatering coupled with filtration maybe the most cost-effective method for microalgal harvesting, but this may be different for different microalgal strains. The comparison also highlights the fact that none of the harvesting methods are cost effective when considering cultivation of microalgae solely for biodiesel production. Hence, it is a necessity to derive a secondary product that has a higher market value when compared with biodiesel. In the past, the majority of studies have focused on freshwater microalgae species and not much work has been done on marine species. With limited availability of freshwater, further research should be focused more on the processing of marine microalgae.

Future perspective

Rapid depletion of fossil fuels and rising GHG emissions have made the case of microalgae as a biofuel source even more compelling. Moreover, microalgae grown on non-arable land have great potential for provision of animal feed, and microalgae can also be used for wastewater purification. At present, harvesting technologies are costly and labour intensive, but recent studies indicate that major efforts are underway to develop new, more efficient and cheaper harvesting technologies, many of which will be microalgal strain specific. Microalgae are being grown in outdoor ponds, greenhouses, photo-bioreactors, fermenters and hybrid systems combining bioreactors and ponds. As more and larger microalgae pilot plants will be in operation within 5–10 years, more accurate economic assessments of different harvesting methods will be possible that will feed into the life cycle analyses of future algal biorefineries. With the availability of new and more efficient harvesting systems, microalgae harvesting will be less costly, easier to manage and more accessible for farmers, rural communities and industry around the world. Microalgal biorefineries are expected to be first established on a large scale in countries with high irradiation, flat, non-arable, desert, saline or low-biodiversity land, and
access to water unsuitable for human consumption or irrigation (brackish, marine or polluted).

References


*  


89. Goh A. *Production of Microalgae Using Pig Waste as a Substrate*. J Cramer, CO, USA

Background

In order to optimise large-scale microalgae cultivation, there was a need to select microalgal species by comparing multiple microalgal strains at lab-scale level. The following screening criteria were used:

1. High growth rate, lipid induction rate.
2. Tolerance to different growth media (fresh water/ marine water).
3. High lipid content.
4. Locally isolated/acquired strain.

To select the ideal candidate for pilot-scale cultivation optimisation tests, different algae strains were grown under controlled identical conditions in the laboratory. The growth curves were obtained, algae were subjected to nutrient starvation stress and lipid content was analysed. Overall, 11 different types of microalgae were chosen based on their rapid growth and potential to accumulate lipids. Out of the 11 strains, 5 strains were characterised in more detail as a part of this PhD study. These were: Chaetoceros muellerii, Chaetoceros calcitrans, Pavlova lutheri, Isochrysis sp. and Tetraselmis sp. M8.

The result for this study was published as a part of a paper entitled: Isolation and Evaluation of Oil-Producing Microalgae from Subtropical Coastal and Brackish Waters. Published in PLoS ONE Journal.

Key findings

Based on the data, the microalgal strain selected for outdoor cultivation was Tetraselmis sp. M8. The reasons for this were:

i. It is a rapidly growing strain with high lipid accumulation potential (high lipid productivity)
ii. It is a locally isolated strain.
iii. The strain belongs to the laboratory (was not purchased).
iv. It is a marine strain and shows a wide salinity tolerance
v. It is motile; as a result it needs less energy to move throughout the culture (less external mixing required).
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 biomass (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-add products such as protein, carbohydrates and a range of fatty acids (FAs). High value omega-3 fatty acids (ω-3) such as eicosapentaenoic (EPA), docosahexanoic (DHA), alpha-linolenic acid (ALA) and arachidonic (AA) are not desirable FAs for biodiesel production. Nevertheless, these ω-3 polyunsaturated fatty acids (PUFAs) are highly valued in human nutrition and therapeutics [15] and are linked to a wide range of cardio and circulatory benefits [16]. Ω-3 fatty acids also play an important role in aquaculture, increasing growth performance and reducing
mortality in the shellfish industry [17,18,19]. This ability to produce value-adding products in addition to biodiesel is important to reduce production cost and make large-scale production viable.

The inherent advantages of a microalgal fuel source are unfortunately offset by current limitations to economically produce it on a large-scale. For example, the cost for obtaining dry biomass, large hexane requirements and limited hexane recycling capacity are currently hindering economic viability. It was estimated that the current cost of producing 1 tonne of microalgal biomass with an average 55% (w/w Dry Weight) oil content needs to be reduced by 10-fold in order to be competitive with petroleum diesel [8]. Furthermore, despite estimates that suggest microalgal oil production (US$9-$25/gallon in ponds, $15-$40 in photobioreactors) could be cheaper than the current price of oil [20], companies commercially producing microalgae have not been able to achieve the predicted yields and production costs. Typical lipid yields of 10 g m⁻²d⁻¹ (Skye Thomas-Hall, personal communication) are still short of achieving the current best case scenarios of 103 to 134 g m⁻²d⁻¹ [21]. The industry is still in its infancy, although recent research and development efforts by large oil companies (e.g. Exxon, BP, Chevron and Shell) would certainly increase production capacity and decrease production costs.

As large variations (10-50%) in lipid content exist between different species of microalgae [22,23], it is necessary to identify strains with high lipid content and suitable lipid composition. The need for high-yielding microalgae is straightforward, as this directly translates to an overall increase in production, although lipid production during normal growth needs to be distinguished from lipid accumulation in response to adverse conditions (e.g. nutrient starvation). Lipid composition is equally important, as quantitative and qualitative differences in the TAG content of a given species will affect the quality of biodiesel and its ability to meet fuel standards. Fuels with high cetane number fatty acids (e.g. myristic acid, palmitic acid, stearic acid) are desirable [24], as higher cetane fuels have better combustion quality and the right cetane number of biodiesel is required to meet an engine’s cetane rating [25]. Microalgal lipids are mostly polyunsaturated, which have a low cetane number and are more prone to oxidation. This can create storage problems and are thus preferred to be at a minimum level for biodiesel production. Nevertheless, polyunsaturated fatty acids lower the cold filter plugging point (CFPP) of fuel and are crucial in colder climates to enable the biodiesel to perform at lower temperatures [3]. With these factors in mind, an “ideal composition” of fatty acids would consist of a mix
of saturated and monounsaturated short chain fatty acids in order to have a very low oxidative potential whilst retaining a good CFPP rating and cetane number.

To date, research efforts have focused on lipid production of individual species, usually investigating the effects different growth conditions have on lipid production and content [26,27,28,29,30]. Unfortunately, direct comparisons of results between studies are unreliable, given the different growth conditions and experimental parameters of each species and also the different methods used for lipid extraction. There is growing interest to compare lipid content and FA composition of multiple microalgae species [11,31,32,33,34,35]. Several studies have revealed algae genera such as *Tetraselmis*, *Nannochloropsis* and *Isochrysis* to have highest high lipid content, particularly under nutrient-deprived conditions [11,31].

Nutrient deprivation is regarded as an efficient way to stimulate lipid production in microalgae in several microalgae species [11,29,36,37], especially saturated and monosaturated FAs [6,38,39]. Unfortunately, lipid accumulation is often associated with a reduction in biomass, which reduces overall lipid accumulation. A batch culture strategy can be adopted to obtain maximal biomass productivity as well as induction of lipid accumulation through nutrient deprivation. Although a common research practice, only Rodolfi et al. [11] have published lipid profiles of multiple microalgae species in a batch culture setting.

The target of our work was to identify the most effective microalgal TAG producers for biodiesel production using a basic batch culture strategy. Most studies utilize experimental designs that include aeration of media volumes of 1 L to 10 L in order identify microalgae strains with high lipid content [31,32,33,36,40]. To provide a direct comparison between different species, this study evaluated eleven microalgae strains collected from local Australian coastal waterways and other collections that originate in various places in the world. Strains were first characterized by microscopy and partial 18S ribosomal RNA sequencing and total fatty acid methyl ester (FAME) contents were then analyzed via GC/MS, which quantifies the fatty acids in triacylglycerides in each strain, thus providing the most accurate representation of the substrate available for biodiesel production. Using growth rate, FAME productivity and FA composition as criteria, this study identified several algae strains to be suitable for biodiesel, including *Tetraselmis* sp. and *Nannochloropsis* sp. as highly versatile candidate strains for a multiple-product algal biorefinery.
Materials and methods

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°0’32"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⁻²s⁻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 algae-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.

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 (1,200xg, 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**

Algae cultures (4 mL each) were centrifuged at 16,000xg 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 16,000xg 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’-GCGGTAATTCCAGCTCAATAGC–3’ and Reverse: 5’-GACCATACTCCCCGGAACC-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
Identification of microalgae and phylogenetic analysis

Nucleotide sequences were obtained from the NCBI database based on the BLAST results of each algae 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 *Tetraselmis* sp. M8, *Chlorella* sp. BR2 and *Nannochloropsis* 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.

**Table 1. Sources and 18S rRNA sequence accessions of microalgae strains used in this study.**

<table>
<thead>
<tr>
<th>Species</th>
<th>Genbank Accession</th>
<th>Location of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Tetraselmis</em> sp. M8</td>
<td>JQ423158</td>
<td>Maroochydore, Qld, Australia</td>
</tr>
<tr>
<td><em>Tetraselmis</em> <em>chui</em></td>
<td>JQ423150</td>
<td>East Lagoon, Galveston, TX, USA</td>
</tr>
<tr>
<td><em>Tetraselmis</em> <em>suecica</em></td>
<td>JQ423151</td>
<td>Brest, France</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp. BR2</td>
<td>JQ423160</td>
<td>Brisbane River, Brisbane, Australia</td>
</tr>
<tr>
<td><em>Dunaliella</em> <em>salina</em></td>
<td>JQ423154</td>
<td>Alice Springs, NT, Australia</td>
</tr>
<tr>
<td><em>Chaetoceros</em> <em>calcitrans</em></td>
<td>JQ423152</td>
<td>Unknown</td>
</tr>
<tr>
<td><em>Chaetoceros</em> <em>muelleri</em></td>
<td>JQ423153</td>
<td>Oceanic Institute, Hawaii, USA</td>
</tr>
<tr>
<td><em>Pavlova</em> <em>salina</em></td>
<td>JQ423155</td>
<td>Sargasso Sea</td>
</tr>
<tr>
<td><em>Pavlova</em> <em>lutheri</em></td>
<td>JQ423159</td>
<td>Unknown location, UK</td>
</tr>
<tr>
<td><em>Isochrysis</em> <em>galbana</em></td>
<td>JQ423157</td>
<td>Unknown location, UK</td>
</tr>
<tr>
<td><em>Chlorella</em> <em>sp.</em> BR2</td>
<td>JQ423156</td>
<td>Brisbane River, Brisbane, Australia</td>
</tr>
</tbody>
</table>

Analytical methods

Measurement of nitrate and phosphate levels in the photobioreactor was performed using colorimetric assays (API, Aquarium Pharmaceuticals and Nutrafin, respectively). Growth rate, doubling time and lipid productivity were calculated as follows. The average growth rate was calculated using the equation $\mu = \ln(N_y/N_x)/(t_y-t_x)$ 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 $T = (t_y-t_x)/\log_2 (N_y/N_x)$ 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 ($\mu g \text{ mL}^{-1} \text{ day}^{-1}$) was calculated as total lipid content ($\mu g/mL$) over the duration of the entire batch culture (laboratory cultures – 9 days, outdoor culture – 12 days).

Microscopic analyses

After a lipid induction phase, microalgae cells were stained with 2 $\mu g/mL$ 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 1000x 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 1.3x10⁶/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 dependant 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 QSST, Bundaberg. Visual microscopy (Figure 1) confirmed the isolation of uniclonal 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).

Table 2: Growth rate analysis of eleven microalgae strains during growth phase (7 days) of batch culture.

<table>
<thead>
<tr>
<th>Species</th>
<th>$\mu_{Ave}$</th>
<th>$\mu_{Exp}$</th>
<th>Day of $\mu_{Exp}$</th>
<th>DT$_{Ave}$ [days]</th>
<th>Cell density$_{Max}$ [x10$^6$cells mL$^{-1}$]</th>
<th>Dry weight [g L$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nannochloropsis</em> sp.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR2</td>
<td>0.32</td>
<td>0.62$^{c, d}$</td>
<td>2-4</td>
<td>2.18$^c$</td>
<td>48.4</td>
<td>0.53</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp. M8</td>
<td>0.35</td>
<td>0.93$^{a, b}$</td>
<td>2-4</td>
<td>2.00$^c$</td>
<td>2.07</td>
<td>0.75</td>
</tr>
<tr>
<td><em>T. chui</em></td>
<td>0.35</td>
<td>1.03$^a$</td>
<td>2-4</td>
<td>1.98$^c$</td>
<td>1.56</td>
<td>0.42</td>
</tr>
<tr>
<td><em>T. suecica</em></td>
<td>0.37</td>
<td>0.5$^d$</td>
<td>0-2</td>
<td>1.85$^{b,c}$</td>
<td>1.52</td>
<td>0.73</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>0.30</td>
<td>0.76$^{a, b, c, d}$</td>
<td>2-4</td>
<td>2.31$^c$</td>
<td>2.14</td>
<td>0.37</td>
</tr>
<tr>
<td><em>C. calictrains</em></td>
<td>0.34</td>
<td>0.59$^{c, d}$</td>
<td>0-2</td>
<td>2.03$^c$</td>
<td>4.71</td>
<td>n/a</td>
</tr>
<tr>
<td><em>C. muelleri</em></td>
<td>0.35</td>
<td>0.71$^{a, b, c, d}$</td>
<td>0-2</td>
<td>1.94$^{b,c}$</td>
<td>4.65</td>
<td>0.50</td>
</tr>
<tr>
<td><em>I. galbana</em></td>
<td>0.35</td>
<td>0.61$^{b, c, d}$</td>
<td>0-2</td>
<td>1.96$^{b,c}$</td>
<td>4.45</td>
<td>0.45</td>
</tr>
<tr>
<td><em>P. lutheri</em></td>
<td>0.48$^a$</td>
<td>0.76$^{a, b, c, d}$</td>
<td>0-2</td>
<td>1.45$^a$</td>
<td>3.95</td>
<td>0.45</td>
</tr>
<tr>
<td><em>P. salina</em></td>
<td>0.45$^a$</td>
<td>0.88$^{a, b, c}$</td>
<td>2-4</td>
<td>1.54$^{a,b}$</td>
<td>5.47</td>
<td>1.68</td>
</tr>
<tr>
<td><em>Chlorella</em> sp. BR2</td>
<td>0.34</td>
<td>0.86$^{a, b, c}$</td>
<td>0-2</td>
<td>2.06$^c$</td>
<td>13.8</td>
<td>0.59</td>
</tr>
</tbody>
</table>
**Tetraselmis** sp.M8

<table>
<thead>
<tr>
<th></th>
<th>0.47</th>
<th>0.48</th>
<th>6-7</th>
<th>1.45</th>
<th>1.61</th>
<th>0.58</th>
</tr>
</thead>
</table>

1. Value represents mean of two replicate samples
2. Different letter superscripts down a column indicate significant difference at 95% level (ANOVA, Bonferroni’s test; P<0.05)
3. Mid-scale outdoor culture

---

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. M8 (S, T) and Tetraselmis suecica (U, V). All images were taken at 100x magnification. Bars represent 20 µm.

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. FR865883). 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 microalgae 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.
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.
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.

Figure 4. Growth curves of different microalgae in this study. *C. calcitrans*, *C. muelleri*, *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)).

The highest average growth rate ($\mu_{ave}$) 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 $\mu_{ave}$ of 0.34 μL$^{-1}$. Specific growth rates ($\mu_{exp}$), were also compared with ANOVA, revealing that *T. chui* had the highest $\mu_{exp}$ 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, 3.9 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 C18:2s (11.7%). *D. salina* and *Chlorella* sp. BR2’s FA profile consisted mostly (nearly 90%) of C16, C18 and their unsaturated derivatives. In *T. chui* and *T. suecica*, C16 (35-37%), unsaturated C18s (37-43%) and unsaturated C20s (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%).
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). ¹Mid-scale outdoors culture.

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%). C16 was found to be a major FA (17-37%) in all the strains tested, particularly in *T. chui*, *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. MB. *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.
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).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Nannochloropsis sp. BR2</th>
<th>T. suecica sp. M8</th>
<th>Tetraselmis sp. M8</th>
<th>D. calci-muehlenbeckii sp. trans lerenia</th>
<th>C. chui sp. sp. M8</th>
<th>P. salina sp. sp. M8</th>
<th>P. chlorophila sp.</th>
<th>Chlorella sp.</th>
<th>Tetraselmis sp. M8</th>
<th>Chlorella sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>0.5</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14</td>
<td>3.5</td>
<td>0.9</td>
<td>0.9</td>
<td>0.4</td>
<td>0.6</td>
<td>10.5</td>
<td>11.6</td>
<td>11.6</td>
<td>19.2</td>
<td>14.4</td>
</tr>
<tr>
<td>C15</td>
<td>0.4</td>
<td>0.1</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>C16</td>
<td>33.0</td>
<td>37.3</td>
<td>35.2</td>
<td>22.5</td>
<td>24.7</td>
<td>23.3</td>
<td>33.2</td>
<td>16.4</td>
<td>26.2</td>
<td>25.0</td>
</tr>
<tr>
<td>C16:1</td>
<td>26.8</td>
<td>2.5</td>
<td>2.3</td>
<td>1.1</td>
<td>2.9</td>
<td>34.1</td>
<td>29.7</td>
<td>2.0</td>
<td>19.1</td>
<td>3.6</td>
</tr>
<tr>
<td>C16:2</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
<td>2.5</td>
<td>1.5</td>
<td>2.7</td>
<td>0.9</td>
<td>3.1</td>
<td>3.4</td>
</tr>
<tr>
<td>C16:3</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>2.9</td>
<td>4.0</td>
<td>5.5</td>
<td>-</td>
<td>-</td>
<td>7.8</td>
<td>0.1</td>
</tr>
<tr>
<td>C16:4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C17</td>
<td>0.4</td>
<td>0.1</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
<td>1.6</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
</tr>
<tr>
<td>C18</td>
<td>3.0</td>
<td>9.0</td>
<td>8.8</td>
<td>3.0</td>
<td>5.8</td>
<td>5.1</td>
<td>4.5</td>
<td>4.4</td>
<td>8.3</td>
<td>9.7</td>
</tr>
<tr>
<td>C18:1</td>
<td>6.0</td>
<td>13.8</td>
<td>15.3</td>
<td>9.1</td>
<td>5.6</td>
<td>5.8</td>
<td>1.7</td>
<td>21.7</td>
<td>1.3</td>
<td>20.0</td>
</tr>
<tr>
<td>C18:2</td>
<td>0.9</td>
<td>8.8</td>
<td>19.7</td>
<td>11.7</td>
<td>7.6</td>
<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
<td>1.1</td>
<td>7.9</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.4</td>
<td>15.1</td>
<td>8.8</td>
<td>28.9</td>
<td>33.8</td>
<td>0.0</td>
<td>0.4</td>
<td>3.1</td>
<td>0.1</td>
<td>1.3</td>
</tr>
<tr>
<td>C18:4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.1</td>
<td>-</td>
<td>12.7</td>
</tr>
<tr>
<td>C20</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>C20:1</td>
<td>-</td>
<td>1.8</td>
<td>2.1</td>
<td>0.1</td>
<td>-</td>
<td>5.9</td>
<td>0.1</td>
<td>-</td>
<td>0.8</td>
<td>4.6</td>
</tr>
<tr>
<td>C20:4</td>
<td>5.9</td>
<td>2.6</td>
<td>3.3</td>
<td>3.4</td>
<td>-</td>
<td>0.9</td>
<td>1.4</td>
<td>13.9</td>
<td>6.1</td>
<td>-</td>
</tr>
<tr>
<td>C20:5</td>
<td>18.8</td>
<td>7.2</td>
<td>2.9</td>
<td>10.6</td>
<td>1.2</td>
<td>12.7</td>
<td>14.0</td>
<td>0.0</td>
<td>21.8</td>
<td>16.1</td>
</tr>
<tr>
<td>C22</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22:4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22:6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>11.8</td>
<td>7.3</td>
<td>10.5</td>
<td>-</td>
</tr>
<tr>
<td>Total saturated (%)</td>
<td>40.7</td>
<td>47.9</td>
<td>45.6</td>
<td>30.4</td>
<td>31.4</td>
<td>40.5</td>
<td>44.0</td>
<td>39.9</td>
<td>41.1</td>
<td>53.0</td>
</tr>
<tr>
<td>Total monounsaturated (%)</td>
<td>32.8</td>
<td>18.2</td>
<td>19.7</td>
<td>10.2</td>
<td>8.6</td>
<td>40.0</td>
<td>31.4</td>
<td>29.6</td>
<td>20.5</td>
<td>14.4</td>
</tr>
<tr>
<td>Total polyunsaturated (%)</td>
<td>26.5</td>
<td>34.0</td>
<td>34.7</td>
<td>59.5</td>
<td>60.0</td>
<td>19.5</td>
<td>24.6</td>
<td>30.5</td>
<td>38.3</td>
<td>41.4</td>
</tr>
<tr>
<td>Total FAMEs (μg mL⁻¹)</td>
<td>56.1</td>
<td>13.5</td>
<td>13.4</td>
<td>18.7</td>
<td>43.0</td>
<td>29.0</td>
<td>29.5</td>
<td>17.6</td>
<td>19.0</td>
<td>31.4</td>
</tr>
<tr>
<td>Total FAME/dry weight (%)</td>
<td>10.6</td>
<td>3.2</td>
<td>10.8</td>
<td>2.5</td>
<td>11.4</td>
<td>-</td>
<td>5.9</td>
<td>3.9</td>
<td>4.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

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 x 10⁶ cells mL⁻¹ on day 7, eventually arriving at 2.3 x 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 significant 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, micro-oxic 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 uni-clonal microalgae 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 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 Supplementary Table S1). Both average growth rate ($\mu_{ave}$) and specific growth rate ($\mu_{exp}$) of the 11 analyzed microalgae strains were calculated from cell count growth curves (Figures 3-4). Overall, $\mu_{ave}$ found in the present study were similar or higher than $\mu_{ave}$ published by [36] and [34], aside from [32] which had nearly twice the $\mu_{ave}$ (Table 4). The specific growth rate ($\mu_{exp}$) 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 $\mu_{exp}$ 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, CO$_2$ 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 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, KNO$_3$ 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 $\mu_{exp}$. *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 microalgal 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.

Table 4: Comparison of FAME productivity (μg mL\(^{-1}\) day\(^{-1}\)) of present study microalgae with lipid productivity of microalgal species from other references.

<table>
<thead>
<tr>
<th>Species</th>
<th>Lipid productivity [μg mL(^{-1}) day(^{-1})]</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nannochloropsis</em> sp. BR2</td>
<td>6.2</td>
<td>This study</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp.</td>
<td>4.6</td>
<td>Huerlimann et al. (2010)(^{12h})</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp.</td>
<td>48.0</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp.</td>
<td>37.6</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp.</td>
<td>60.9</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> oculata</td>
<td>10.0</td>
<td>Converti et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp. M8</td>
<td>2.1</td>
<td>This study</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp. M8 (outdoor)</td>
<td>4.8</td>
<td>This study</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp.</td>
<td>18.6</td>
<td>Huerlimann et al. (2010)(^{12h})</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp.</td>
<td>43.4</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp.</td>
<td>10.7</td>
<td>Patil et al. (2007)(^{GCM, 24h, CO2})</td>
</tr>
<tr>
<td><em>Tetraselmis</em> chui</td>
<td>1.5</td>
<td>This study</td>
</tr>
<tr>
<td><em>Tetraselmis</em> suecica</td>
<td>27.0</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Tetraselmis</em> suecica</td>
<td>1.5</td>
<td>This study</td>
</tr>
<tr>
<td><em>Dunaliella</em> salina</td>
<td>36.4</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Dunaliella</em> salina</td>
<td>33.5</td>
<td>Takagi et al. (2006)</td>
</tr>
<tr>
<td><em>Chaetoceros</em> mulleri</td>
<td>3.3</td>
<td>This study</td>
</tr>
<tr>
<td><em>Chaetoceros</em> mulleri</td>
<td>21.8</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Chaetoceros</em> calcitran</td>
<td>3.2</td>
<td>This study</td>
</tr>
<tr>
<td><em>Chaetoceros</em> calcitran</td>
<td>17.6</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Chaetoceros</em> sp.</td>
<td>16.8</td>
<td>Renaud et al. (2002)(^{12h})</td>
</tr>
<tr>
<td><em>Isochrysis</em> galbana</td>
<td>2.0</td>
<td>This study</td>
</tr>
<tr>
<td><em>Isochrysis</em> sp.</td>
<td>24.9</td>
<td>Renaud et al. (2002)(^{12h})</td>
</tr>
<tr>
<td><em>Isochrysis</em> sp.</td>
<td>12.7</td>
<td>Huerlimann et al. (2010)(^{12h})</td>
</tr>
<tr>
<td><em>Isochrysis</em> sp.</td>
<td>37.7</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td>I. galbana</td>
<td>12.4</td>
<td>Patil et al. (2007)(^{GCM, 24h, CO2})</td>
</tr>
<tr>
<td><em>Pavlova</em> lutheri</td>
<td>2.0</td>
<td>This study</td>
</tr>
<tr>
<td><em>Pavlova</em> lutheri</td>
<td>50.2</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Pavlova</em> salina</td>
<td>2.1</td>
<td>This study</td>
</tr>
<tr>
<td><em>Pavlova</em> salina</td>
<td>49.4</td>
<td>Rodolfi et al. (2009)(^{24h, CO2})</td>
</tr>
<tr>
<td><em>Pavlova</em> sp.</td>
<td>21.7</td>
<td>Patil et al. (2007)(^{GCM, 24h, CO2})</td>
</tr>
</tbody>
</table>
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 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* CS-49 and *C. calcitrans* CS-178 were studied under different conditions to reveal significantly different results. It should be noted that the conditions of the current experimental design were not meant to achieve maximum lipid production but to determine the best lipid producing candidates under standard “unoptimized lab conditions”, which were *Nannochloropsis* sp. BR2, *D. salina* and *Chlorella* sp. BR2. Higher confidence in the data may be obtained by growing cultures completely independently (i.e. experiments carried out separately at different times with a different culture). Subsequent studies may focus on the comparison of best strains under fully optimized and/or large-scale commercial conditions. In our study, *Tetraselmis* sp M8 was chosen for a scale-up study based on its fast growth rates,
culture dominance and ease of harvesting by settling. A comparison of the indoor laboratory conditions to mid-scale (1000 L) outdoor conditions showed that lipid productivity more than doubled under these conditions. Although further long-term studies will be required, these preliminary findings demonstrate the potential for optimization and emphasize that outdoor and large-scale conditions differ strongly from laboratory conditions.

Suitable candidates for biodiesel production require not only high lipid productivity, but also suitable FA content. Recommended FAs for good biodiesel properties 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 mediocre lipid productivity 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 despite 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) that would allow for a biorefinery approach to biodiesel production. It should be noted, however, that microalgal 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 microalgal biodiesel would require a biorefinery approach to produce biodiesel as well as other value-added products such ω-3 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 [31,35,57].

The use of a nutrient starvation phase to improve TAG productivity (particular 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. tetrathele* [40], which may explain the high levels of PUFA observed in *Tetraselmis* sp.

In a 1000 L-outdoor setting, *Tetraselmis* sp. M8 was found to have an increased $\mu_{Ave}$ 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.

**Acknowledgements**

We wish to thank Tania Catalina Adarme-Vega, Kalpesh Sharma, Felicitas Vernen, Holger Schuhmann, Bart Nijland, Priyanka Nayak, Yamini Kashimshetty, Ekaterina Novak, Miklos Deme and Bernie Degnan for technical assistance and useful discussions. We are also grateful to QSST and CSIRO for provision of additional microalgae strains.

**References**


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).

<table>
<thead>
<tr>
<th>Species</th>
<th>Growth rate</th>
<th>Lipid productivity</th>
<th>References</th>
<th>Air supply</th>
<th>Light cycle</th>
<th>Light intensity</th>
<th>Volume</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nannochloropsis</em> sp. I</td>
<td>0.3</td>
<td>6.2</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>20 mL</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp. II</td>
<td>0.5</td>
<td>4.6</td>
<td>Huerlimann et al. (2010)</td>
<td>Air</td>
<td>12L:12D</td>
<td>250 μmol photons m(^{-2}) s(^{-1})</td>
<td>10 L</td>
<td>F/2</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp. III</td>
<td>0.7</td>
<td>18.2</td>
<td>Rodolfi et al. (2007)</td>
<td>Air/CO(_2) (55%)</td>
<td>24L:00</td>
<td>100 μmol photons m(^{-2}) s(^{-1})</td>
<td>100 mL</td>
<td>F</td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp. IV</td>
<td>0.9</td>
<td>10.9</td>
<td>Rodolfi et al. (2008)</td>
<td>Air/CO(_2) (55%)</td>
<td>24L:00</td>
<td>100 μmol photons m(^{-2}) s(^{-1})</td>
<td>100 mL</td>
<td>F</td>
</tr>
<tr>
<td><em>N. oceanica</em></td>
<td>0.07</td>
<td>10.0</td>
<td>Conversi et al. (2009)</td>
<td>sec. air &amp; CO(_2)</td>
<td>12L:20</td>
<td>70 μmol m(^{-2}) s(^{-1})</td>
<td>2 L</td>
<td>F/2</td>
</tr>
<tr>
<td><em>Trachelomonas</em> sp. M1</td>
<td>0.35</td>
<td>2.1</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>20 mL</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td><em>T. suecica</em> sp. M2</td>
<td>0.60</td>
<td>4.6</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>1000 mL</td>
<td>F/2</td>
<td></td>
</tr>
<tr>
<td><em>T. suecica</em> sp. M3</td>
<td>0.1</td>
<td>14.6</td>
<td>Huerlimann et al. (2010)</td>
<td>Air</td>
<td>12L:12D</td>
<td>250 μmol photons m(^{-2}) s(^{-1})</td>
<td>10 L</td>
<td>F/2</td>
</tr>
<tr>
<td><em>T. suecica</em> sp. M4</td>
<td>0.0</td>
<td>4.4</td>
<td>Rodolfi et al. (2009)</td>
<td>Air/CO(_2) (55%)</td>
<td>24L:00</td>
<td>100 μmol PAR photons m(^{-2}) s(^{-1})</td>
<td>100 mL</td>
<td>F</td>
</tr>
<tr>
<td><em>T. suecica</em> sp. M5</td>
<td>0.1</td>
<td>10.7</td>
<td>Pari et al. (2016)</td>
<td>Air &amp; CO(_2)</td>
<td>24L:00</td>
<td>11 W</td>
<td>1.3 L</td>
<td>F/2</td>
</tr>
<tr>
<td><em>T. chui</em></td>
<td>0.55</td>
<td>1.5</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>20 mL</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td><em>T. chui</em></td>
<td>0.3</td>
<td>2.7</td>
<td>Rodolfi et al. (2009)</td>
<td>Air/CO(_2) (55%)</td>
<td>24L:00</td>
<td>100 μmol PAR photons m(^{-2}) s(^{-1})</td>
<td>100 mL</td>
<td>F</td>
</tr>
<tr>
<td><em>T. suecica</em></td>
<td>0.5</td>
<td>1.5</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>20 mL</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td><em>T. suecica</em></td>
<td>0.3</td>
<td>3.4</td>
<td>Rodolfi et al. (2009)</td>
<td>Air/CO(_2) (55%)</td>
<td>24L:00</td>
<td>100 μmol PAR photons m(^{-2}) s(^{-1})</td>
<td>100 mL</td>
<td>F</td>
</tr>
<tr>
<td><em>T. suecica</em></td>
<td>0.5</td>
<td>-</td>
<td>Lee et al. (2015)</td>
<td>Air</td>
<td>12L:12D</td>
<td>150 μmol photons m(^{-2}) s(^{-1})</td>
<td>10 L</td>
<td>F/2</td>
</tr>
<tr>
<td><em>C. reinhardtii</em></td>
<td>0.2</td>
<td>1.3</td>
<td>de la Rosa &amp; Vilaques (2009)</td>
<td>Air</td>
<td>24L:12D</td>
<td>100 μmol PAR photons m(^{-2}) s(^{-1})</td>
<td>1 L</td>
<td>F</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>0.32</td>
<td>4.8</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>20 mL</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>0.4</td>
<td>-</td>
<td>Rajah et al. (2007)</td>
<td>-</td>
<td>12L:12D</td>
<td>30 μmol photons m(^{-2}) s(^{-1})</td>
<td>100 mL</td>
<td>F/2</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>0.3</td>
<td>-</td>
<td>Garcia et al. (2007)</td>
<td>Air</td>
<td>12L:12D</td>
<td>100 μmol photons m(^{-2}) s(^{-1})</td>
<td>1 L</td>
<td>F</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>0.5</td>
<td>3.5</td>
<td>Takagi et al. (2006)</td>
<td>Air/CO(_2) (55%)</td>
<td>24L:00</td>
<td>100 μmol PAR photons m(^{-2}) s(^{-1})</td>
<td>500 mL</td>
<td>F</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>0.2</td>
<td>-</td>
<td>Garcia et al. (2007)</td>
<td>Air</td>
<td>12L:12D</td>
<td>80 μmol photons m(^{-2}) s(^{-1})</td>
<td>1 L</td>
<td>J</td>
</tr>
<tr>
<td><em>C. musciformis</em></td>
<td>0.32</td>
<td>3.3</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>20 mL</td>
<td>F</td>
<td></td>
</tr>
<tr>
<td><em>Chlorella reinhardtii</em></td>
<td>0.34</td>
<td>6.3</td>
<td>Rodolfi et al. (2009)</td>
<td>Air/CO(_2) (55%)</td>
<td>24L:00</td>
<td>100 μmol PAR photons m(^{-2}) s(^{-1})</td>
<td>100 mL</td>
<td>F</td>
</tr>
<tr>
<td><em>E. caldarora</em></td>
<td>0.54</td>
<td>5.2</td>
<td>This study</td>
<td>12L:12D</td>
<td>120 μmol photons m(^{-2}) s(^{-1})</td>
<td>20 mL</td>
<td>F</td>
<td></td>
</tr>
</tbody>
</table>
Background

Once the algae were screened and the desired microalgal strain was selected it was important to choose and optimise the cultivation system. Different cultivation systems have different advantages and it is vital to use a cultivation system with low set-up and running costs and that is scalable. In order to find the optimum cultivation system, three different cultivation systems were tested over the period of 33 days. The systems used for this study were designed and constructed on the roof of the Goddard building (8) of the University of Queensland, St Lucia campus. The systems used as part of this PhD study were:

1. Open system (raceway ponds)
2. Closed system (photo-bioreactor)

The two systems were compared on the basis of algae growth rate, doubling time and average growth rate to total solar exposure per growing cycle. The aim of this part of the study was to identify the cultivation system with the highest growth rate and shortest doubling time. By the end of the study, the data was compared with other studies performed using hybrid cultivation systems. Also this study has been carried out to determine if pressurised air can be used as a mixing tool instead of a traditional paddle wheel.

The result for this study will be published as a part of a paper entitled: Comparison of microalgae cultivation in photo-bioreactor, open raceway pond and a two-stage hybrid system. Paper ready for submission.

Key findings:

- A hybrid microalgae cultivation system performed better than a single cultivation system (raceway or photo-bioreactor).
- By the end of this study the use of pressurised air as a mixing tool instead of a traditional paddle wheel was found to be more efficient in terms of removing dissolved oxygen from the system.
COMPARISON OF MICROALGAE CULTIVATION IN PHOTOBIOREACTOR, OPEN RACEWAY POND AND A TWO-STAGE HYBRID SYSTEM

Sourabh Garg¹, Rakesh R Narala¹, Yan Li, Skye R Thomas-Hall, Kalpesh K Sharma, Miklos Deme, Peer M Schenk*

Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

¹These authors contributed equally. *Corresponding author email: p.schenk@uq.edu.au

Abstract

In the wake of intensive fossil fuel usage and CO₂ accumulation in the environment, research is targeted towards sustainable alternate bioenergy that can suffice the growing need for fuel and also that leaves a minimal carbon footprint. Biodiesel production from microalgal lipids can potentially be carried out more efficiently, leaving a smaller footprint and without competing for arable land or biodiverse landscapes. However, current algae cultivation systems and lipid induction processes must be significantly improved and are threatened by contamination with other algae or algal grazers. To address this issue, we have developed an efficient two-stage cultivation system using the marine microalga Tetraselmis sp. M8. This hybrid system combines exponential biomass production in positive pressure air lift-driven bioreactors with a separate synchronized high lipid induction phase in nutrient deplete open raceway ponds. A comparison to either bioreactor or open raceway pond cultivation system suggests that this process displayed significantly higher growth rates and potentially leads to higher productivity of lipid-enriched algal biomass. Nutrients are only added to the closed bioreactors and open raceway ponds have turnovers of only a few days, thus avoiding the critical issue of contamination.

Introduction

Microalgae are considered a promising feedstock for next-generation biofuel production because they are potentially 10-20 times more productive than any other biofuel crop and their large-scale cultivation does not need to compete for arable land or precious biodiverse landscapes [1-4]. Importantly, they are also able to grow in saline and even wastewater [5, 6]. However, commercial cultivation of microalgae for biodiesel, a relatively low value product, does not appear economically feasible with the current microalgal cultivation and harvesting techniques [7, 8].
Microalgae accumulate large amounts of lipid bodies containing triacylglycerides under adverse conditions, such as during nutrient deprivation [9]. Under these circumstances, microalgae stop dividing but are still able to perform photosynthesis and the accumulation of triacylglycerides is considered a survival strategy to endure adverse conditions [10, 11]. Cultivation of microalgae for biodiesel production, however, aims at maximizing lipid productivity (or lipid yields) which takes both, growth rates and lipid contents, into consideration. In batch cultivation systems, microalgae are first grown exponentially to increase their biomass which is then followed by a lipid induction process, usually by omitting nutrient supply towards the end of the growth phase. Other lipid induction techniques are also available and their combination may lead to improved lipid contents [12].

The two most common methods of microalgae cultivation are open cultivation systems such as open ponds, tanks and raceway ponds and controlled closed cultivation systems using different types of bioreactors. One of the first attempts to scale up and cultivate microalgae was achieved using open raceway ponds [13]. Since then, extensive research has been carried out to cultivate microalgae in open cultivation systems. Some of the major advantages of an open cultivation system are minimal capital and operating costs, and a lower energy requirement for culture mixing. On the downside, open systems require large areas to scale up and are susceptible to contamination and bad weather. Also, it is difficult to have control over growth parameters such as evaporation, culture temperature, etc. [3, 14, 15].

Closed cultivation systems, such as photobioreactors (PBRs), are more efficient in terms of quality as they can be operated at highly controlled conditions and therefore can overcome the disadvantages of an open cultivation system. PBRs can be designed and optimized in accordance with the strain of choice. This closed system utilizes relatively less space while increasing the light availability and greatly decreasing cross contamination issues. However, PBRs also have some disadvantages, such as bio-fouling, overheating, and built-up of dissolved oxygen resulting in growth limitation and more importantly very high capital costs for designing and operating [16, 17].

The design and principle of cultivation systems change based on the specific needs [18]. Open ponds built in a wastewater treatment plant can be circular in shape or driven by gravity flow. Similarly, the basic tubular design of the PBRs has been improved over the past decade to facilitate better light availability and culture mixing to produce a range of
pharmaceutical products to high-value nutritional products. PBRs were found to be more efficient when operated with continuous cultures [3, 19]. Continuous cultures in closed systems can be used for higher biomass productivity but cannot be used for lipid induction by stress mechanisms (nutrient starvation) to produce biodiesel.

Although extensive studies have been carried out on open and closed cultivation methods, very little work has been carried out on two-stage hybrid cultivation systems. Two-phase hybrid cultivation systems have been proposed as an advantageous microalgae cultivation system, as they are able to essentially separate biomass growth from the lipid accumulation phase [20-23]. Recently, a life cycle analysis demonstrated a considerably reduced environmental impact when comparing various open and closed cultivation systems with hybrid cultivation [24]. To test whether productivity may also vary for these different systems, a side-by-side comparison was carried out in the present study, using the same algal culture. A PBR was used for continuous growth phase of the microalgae culture while an open raceway pond was used for stress induction and synchronized lipid accumulation. A pilot-scale hybrid cultivation system has been constructed where microalgal growth for lipid production was compared to cultivation in either closed air lift-driven tubular PBR or open raceway ponds. The proposed hybrid cultivation system allows for a separate lipid accumulation phase where one or more efficient stress induction techniques can be carried out, while effectively avoiding the issue on contamination. Key parameters sunlight, nutrients, CO2 and water that affect outdoor cultivation of microalgae were examined and carefully monitored over time to understand the importance of these various factors for growth and lipid productivity.

Materials and Methods

Algae culturing and analyses

Microalgal strain *Tetraselmis* sp. M8 had previously been shown to accumulate significant amounts of lipids after nutrient deprivation [25]. It was collected in an intertidal rock pool at Maroochydore, Australia (26°39′39″S 153°6′18″E). Pure *Tetraselmis* sp. M8 cultures were grown in F/2 medium (AlgaBoost™) in autoclaved seawater (collected at Cleveland, Brisbane, Australia). Laboratory culturing conditions were described previously [25].

A haemocytometer (Bright Line, Sigma) was used to count the algae cells manually. 100 μL of culture was transferred into an Eppendorf tube and 0.1 μL of acetic acid was added
to ensure the algae lose their motility. Growth rate and doubling time were calculated using the formulae mentioned below

\[
\text{Growth rate (K')} = \frac{\ln \left( \frac{N_2}{N_1} \right)}{t_2 - t_1}
\]

\[
\text{Doubling time} = \frac{1}{k \cdot \ln(2)}
\]

Where, \( K' \) = growth rate,

\( N_1 \) and \( N_2 \) = biomass at time1 \( (t_1) \) and time2 \( (t_2) \) respectively.

Nitrate and phosphate concentrations were measured in seawater using API Nutrient testing kits according to the manufacturer’s instructions.

**Culture scale-up and monitoring**

A 50 mL preculture of pure *Tetraselmis* sp. M8 was used to inoculate a 2 L glass bottle and culture was made up to one litre with fresh autoclaved seawater containing F/2 medium. Filtered air was supplied through a Millipore syringe filter for uniform mixing of the culture and to prevent stagnation at the bottom of the bottle. The set-up was undisturbed for 4 d. On the end of the fourth day, 250 mL of the culture was transferred into a clear hanging polyethylene bag (80 cm x 50 cm) which contained 4.75 L of fresh F/2 medium in autoclaved seawater. Filtered air was supplied through a Millipore filter for mixing. The set up was undisturbed for four days.

Four polyethylene bags were set up under outdoor conditions (roof structure of a 3-storey building; University of Queensland, St Lucia campus, building 8) and were each filled with 19 L of fresh F/2 medium in seawater. The 5 L culture grown in the lab was mixed uniformly and 1 L was added to each 19 L bag. These outdoor cultures were left under direct sunlight for 4 d to achieve maximum cell density. The algal cultures of the four bags were then used for inoculation of a closed PBR which contained 1,200 L fresh F/2 medium in seawater. CO\(_2\) was supplied to the PBR to control the pH at around 8.4 using a Weipro pH 2010 controller. This culture served as the starting culture for all subsequent experiments described in the Results.
Cell densities and nutrients were monitored on a daily basis using 5 mL samples of culture from the closed PBR or raceway ponds. Daily sampling was carried out at 4.00 pm (AEST). F/2 nutrients were added when required (nutrients depleted to less than 10%). In the PBR and raceway pond, the starvation phase was started once the cells attained a density of \(1.5 \times 10^6\) cells/mL. Half the volume was harvested after the nutrients were completely used and the cell density exceeding \(2.0 \times 10^6\) cells/mL. In hybrid system cultivation, when the desired PBR cell density of over \(2 \times 10^6\) cells/mL was reached, typically half the volume of the PBR (600 L) was transferred into the open raceway pond for lipid induction for 3-4 days and the removed volume of the PBR was replaced with fresh F/2 medium in seawater. Apart from this sampling, climate data was obtained from the Australia’s Bureau of Meteorology, including temperature and solar exposure [26]. PBR and raceway pond cultivation systems were tested simultaneously from 20/5/2011 to 21/6/2011. Both cultivation systems were cleaned and sanitized with bleach before cultivation. Hybrid system cultivation was performed from 05/07/2011 to 8/08/2011. Cell count, nutrient concentrations (nitrate and phosphate), temperature and solar exposure values were plotted on graphs for the open raceway pond, the closed PBR and the hybrid cultivation system. To avoid biofilm formation in the PBR that could cause light limitation, the polyethylene tubes were occasionally (once a week) slapped to loosen any benthic cells. Nile red staining was performed as described previously [25].

Results

Design and construction of pilot-scale photobioreactor and raceway ponds

A closed PBR and an open raceway pond were designed for side-by-side pilot-scale outdoor algae cultivation using an airlift mechanism for mixing. The volume of the PBR was approximately 1,200 L and the raceway pond held 1,000 L, as shown in Figures 1 and 2. The surface area of the PBR and each raceway pond was 4.6 m² and 6 m² respectively. For a direct comparison of both cultivation systems, PBR and an open raceway pond were used for side-by-side algae cultivation. This included cycles of growth and lipid induction before harvesting of biomass. Synchronized lipid induction was verified by Nile red staining before harvesting (Figure 3). The following paragraphs describe the cultivation cycles applied and monitored for each cultivation system:
Figure 1. Design and specifications of pilot-scale (A) tubular photobioreactor (1:35 scale) and (B) two open raceway ponds (1:75 scale). Mixing was achieved by aeration with pressurized air using an airlift (aeration disc with 6,000 exit holes for the PBR and a single exit point at a lowered section of the raceway ponds).
Outdoor photobioreactor cultivation

Cell counts (cells/mL) and all other parameters of PBR-grown *Tetraselmis* sp. M8 culture were recorded for 33 days (Figure 4). The first cycle started growing exponentially after day 6 and nutrient stress set in on day 8. Nile red staining was performed on the following days to monitor the lipid induction. Low sunlight was recorded on day 10 followed by a decrease in lipids and cell numbers on day 11. Fresh F/2 medium was added on day 12 and half of the culture volume (600 L) was harvested on day 13. Similarly, nutrient stress
set in on day 20 during the second growth cycle and half of the culture was harvested again on day 23. The third growth cycle achieved maximum cell density on day 32 with accumulated lipid content, but declined in cell numbers on day 33. The entire culture was harvested.

**Open raceway pond cultivation**

Cultivation in the raceway pond was carried out in parallel to PBR cultivation and led to three harvesting events on day 13, 23 and day 33 over the same time period (Figure 5). Similar to the first growth cycle in the PBR, nutrient stress was measured on day 8 and the culture reached a density of 2 million cells per mL followed by a decline in density on day 12. F/2 nutrients were added and half the volume (500 L) was harvested. On day 23, the cell density reached 2.3 million cells per mL after nutrient stress from day 20 to day 22. Maximum cell densities were observed on days 27 and 28. The culture was nutrient-stressed during the following two days and the cell density was 2.8 million cells per mL on day 32 with substantial lipid accumulation. Hence, all the culture was harvested.

**Two-stage hybrid cultivation system**

A two-stage hybrid cultivation system was applied where a portion of rapidly growing cells are transferred from the PBR to open raceway ponds where nutrients diminish and algae are harvested upon lipid accumulation. Figure 6 shows the cell density and various harvesting points in the PBR as part of the two-stage hybrid cultivation approach. During these harvesting events, at least half of the PBR culture volume was transferred to one of the raceway ponds, where lipid biosynthesis and accumulation was stimulated by nutrient depletion. The initial cell concentration of the PBR was 1.2 million cells per mL. Initially it took 8 days for the medium to get exhausted and during this cycle the highest cell density was monitored (up to 3 million cells per mL). On day 8, based on the high cell density, 900 L of the culture was transferred into the raceway pond for lipid induction. The PBR was refilled with medium for the second cycle. The duration of the second cycle was 7 days in the PBR followed by raceway pond cultivation for lipid induction. For the third cycle, as the cell density was 1.9 million cells per mL and the nutrient concentration was below detection limit; half (600 L) of the culture was transferred into the raceway pond after only 4 days. During the last three cycles, microalgae were cultured in the PBR for 5, 6 and 6 days, respectively, followed by 3-4 days each of starvation in the raceway pond.
Figure 4. Monitoring of photobioreactor cultivation for 33 days. (A) Daily global solar irradiance. (B) Minimum and maximum air temperature recorded for the respective day. (C) Cells (10^4) per mL. White and red colours represent the start and finish of each cycle, respectively.
Figure 5. Monitoring of raceway pond cultivation for 33 days. (A) Daily global solar irradiance. (B) Minimum and maximum air temperature recorded for the respective day. (C) Cells ($10^4$) per mL. White and red colours represent the start and finish of each cycle, respectively.
Comparison of individual open pond or PBR cultivation with two-stage hybrid cultivation system

Both, PBR and open raceway pond cultivation, resulted in three main growth cycles and harvesting events (Figures 4 + 5). During the same amount of time the hybrid cultivation system led to six main growth cycles and harvesting events (Figure 6). Accordingly, the average growth rate of the hybrid system was significantly higher than that of both single systems (Figure 7A). The main reason for this appears to be that biomass growth and lipid induction phases are essentially independent from each other when using the hybrid cultivation system. This enables to keep the culture in exponential growth at a very high
cell density. On the other hand, algal cultures in individual cultivation systems (PBR or open raceway pond) go through phases of exponential growth, nutrient starvation leading to reduced growth rates, followed by a brief lag phase before the next growth phase (Figures 4 + 5). To determine productivity of all systems, biomass harvests (presented as g/m²/d) over the duration of the experiments are shown in Figure 7B.

Figure 7. (A) Growth rates, and (B) areal biomass productivities for different cultivation systems. Different letters show statistically significant differences. ($P<0.05$)
Discussion

In the present study, three growth and harvesting cycles were carried out for the closed PBR and the open raceway pond, while for a similar duration, six cultivation cycles were achieved using the two-stage hybrid cultivation. The higher number of growth and harvesting cycles in the hybrid system was due to the use of separate cultivation systems for biomass growth and lipid induction phases. As the culture in growth phase never ran out of nutrients, two separate systems help maintain higher growth rates and lower the chance of culture dormancy or contamination. It was hypothesized that a separation of growth phase and lipid induction would be advantageous because microalgae typically either divide to increase the cell numbers (usually during ideal nutrient replete conditions) or lipid biosynthesis will be initiated as a means to increase survival capability during adverse conditions such as nutrient deprivation. In the present study, we found that nutrient deplete cultures (lipid accumulation phase) still underwent cell divisions, although at a lower rate. From the results, *Tetraselmis* sp. M8 grown in the hybrid system had the highest average growth rate and thus the lowest doubling time (Figure 7A,B) while resulting in increased biomass harvests (Figure 7B). However the increase was not significant. Based on two independent published studies using the biomass obtained from this study [25, 36], the total TAG productivity (as measured by fatty acid methyl esters) was found to be 10% dry weight of the biomass. Since the average growth rate of hybrid cultivation cannot be directly compared to that of either closed PBR or raceway pond due to different weather conditions, especially solar irradiance, further analysis was carried out to normalize the areal productivity of biomass to the total solar irradiance that occurred during the testing periods (Figure 8). This showed that the cultures in the hybrid cultivation produced significantly more biomass than the raceway cultivation system when normalized to solar exposure. However, results suggest that there was no significant difference in aerial productivity between hybrid system and single stage PBR.
Table 1 provides a comparison of advantages and disadvantages of open ponds, PBRs and two-phase hybrid cultivation systems. These should be considered before proceeding with the construction of either cultivation system. A major advantage for hybrid systems can be expected for longer term cultivation where contamination by other algae or predators becomes a major concern. These problems occur especially in open pond systems or during times of reduced algae growth [3, 35]. During hybrid cultivation in the present study, the continuously-grown culture was grown in a closed PBR which rarely experiences phases of reduced growth or stagnation, while the contamination-prone open ponds only ever held cultures for a few days before being cleaned.

The present study was a pilot study aimed at identifying the most suitable system for high rate algae cultivation for lipid production. Higher yields can be expected if cultivation conditions are further improved, e.g. by automated harvesting based on cell density. For example, it may be advisable to harvest high cell density cultures when irradiance is expected to be low for the following days, to avoid light limitation that may lead to stagnant growth or even cell death. Future studies should also focus on long-term monitoring of these systems and use larger-scale demonstration facilities to enable reduction of operating costs, energy input and environmental impact.
Table 1. Comparison of various algae cultivating methods considering data from the present and previous studies [3, 7, 23, 28-35].

<table>
<thead>
<tr>
<th>Factor</th>
<th>Photobioreactor</th>
<th>Raceway pond</th>
<th>Hybrid system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space required</td>
<td>Moderate</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Evaporation loss</td>
<td>Low</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>CO₂ sparging efficiency</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Maintenance</td>
<td>Difficult</td>
<td>Easy</td>
<td>Moderate</td>
</tr>
<tr>
<td>Contamination risk</td>
<td>Low</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Biomass quality</td>
<td>Reproducible</td>
<td>Variable</td>
<td>Reproducible</td>
</tr>
<tr>
<td>Energy input for mixing</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Operation type</td>
<td>Batch</td>
<td>Batch</td>
<td>Continuous</td>
</tr>
<tr>
<td>Setup cost</td>
<td>High</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Maintaining continuous</td>
<td>Difficult</td>
<td>Difficult</td>
<td>Easy</td>
</tr>
<tr>
<td>exponential phase</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Acknowledgements

We wish to thank Sayli Ghorpade for assistance with graphical representations and the Australian Research Council and North Queensland & Pacific Biodiesel for financial support.

References

6. Abou-Shanab, R. A. I.; Ji, M.-K.; Kim, H.-C.; Paeng, K.-J.; Jeon, B.-H., Microalgal species growing on piggery wastewater as a valuable candidate for nutrient removal...


3. FLOTATION OF MARINE MICROALGAE: EFFECT OF ALGAL HYDROPHOBICITY

Background

Once microalgae are cultivated and the right density is achieved there is a need to separate microalgae from water before they can be used for further processing. This is often referred to as harvesting. Harvesting is one of the major hurdles preventing successful scale-up. Although the selection of suitable algae harvesting techniques depends largely on the microalgal species and the desired final product, several methods have been proposed for algae harvesting, including centrifugation, filtration, membrane separation, sedimentation with flocculation, gravity sedimentation, and froth flotation.

Froth flotation has a high potential to resolve harvesting issues; however, so far it had not been considered for marine species. In the case of marine microalgae often high ionic strength is blamed for poor flotation performance which contradicts the findings from coal flotation studies and no further research was carried out on marine strains, thus making it a promising research area where improvements can be made. In the present study, the effectiveness of flotation on marine microalgae harvesting was investigated. The study was carried out using a mechanical cell on fresh water and marine microalgae to compare the performance of two algae. The major aim of this study was (1) to identify the key factors that play important roles in flotation performance and (2) to determine the feasibility of flotation for marine microalgae.

The result for this study was published as a part of a paper entitled: Flotation of marine microalgae: Effect of algal hydrophobicity. Published in Bioresource Technology.

Key findings:

- Ionic strength played a rather minor role in determining the flotation of *Tetraselmis* sp. M8 and its hydrophobicity.
- Flotation recovery and microalgal hydrophobicity increased with increasing collector dosage.
- Hydrophobicity was identified as the key factor.
FLOTATION OF MARINE MICROALGAE: EFFECT OF ALGAL HYDROPHOBICITY

Sourabh Garg a, Yan Li a, Liguang Wang b, Peer M. Schenk a

a Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane QLD 4072, Australia
b School of Chemical Engineering, The University of Queensland, Brisbane QLD 4072, Australia

Received 6 May 2012, Revised 29 June 2012, Accepted 29 June 2012, Available online 15 July 2012

Abstract

This study aims to understand the underlying reasons for the poor flotation response of marine microalgae. The flotation performance and hydrophobicity of a freshwater microalga (*Chlorella* sp. BR2) were compared to those of a marine microalga (*Tetraselmis* sp. M8) at different salinities in the presence of a cationic collector, tetradecyl trimethylammonium bromide. It was found that microalgal hydrophobicity played a more important role than salinity in determining the flotation performance.

Highlights

- Algal hydrophobicity has a profound impact on microalgae flotation.
- The ionic strength of flotation medium has little impact on microalgae flotation.
- Algal hydrophobicity can be improved by using a cationic collector.

Keywords

Marine microalgae; Froth flotation; Hydrophobicity; Salinity; Cationic surfactant

Introduction

Microalgae are photosynthetic organisms with great potential to harvest sunlight and convert carbon dioxide into biofuels, health food and animal feed (Chisti, 2007 and Walker, 2005). They have a high photosynthetic efficiency, do not need to compete with edible crops and have comparatively higher oil productivity. Microalgae arguably have become the most promising candidate for the production of biodiesel and other high value products (Chisti, 2007, Ota et al., 2009 and Schenk et al., 2008). Biofuel production from microalgae
can be divided into the following major steps: algae cultivation, biomass harvesting/dewatering, oil extraction and oil conversion to biofuel (Ryan, 2009). The operational costs for dewatering contribute from 20% to 30% to the total biofuel production costs (Brennan and Owende 2010). Dewatering is recognized as a major impediment towards the industrial-scale manufacturing of microalgae bio-products (Danquah et al., 2009 and Uduman et al., 2010).

Although the selection of suitable algae harvesting techniques depends largely on the microalgae species and the desired final product, several methods have been proposed for algae harvesting, including centrifugation, filtration, membrane separation process, sedimentation with flocculation, gravity sedimentation, and froth flotation (Phoochinda and White, 2003 and Uduman et al., 2010). However, most of these methods are of low efficiency and have high capital costs and high energy consumption. For example, centrifugation requires high energy input, a huge cost for large-scale processing which may also damage cells due to high shear forces, resulting in a significant loss of the products of interest (Knuckey et al. 2006). Permeable membranes used for filtration and screening are also easily clogged by tiny microalgae (Uduman et al. 2010) and frequent scraping would significantly shorten the lifetime of these membranes, resulting in high operating costs (Molina Grima et al. 2003). Flocculation seems to be a promising approach for large-scale harvesting, but its application appears to be currently limited to freshwater microalgae. As the ionic strength of water increases, the efficiency of flocculating agent decreases (Uduman et al. 2010). Furthermore, depending on the flocculants, its residues in recycled water may inhibit or prevent renewed algae growth.

Flotation is a proven technology to effectively capture small particles up to 500 µm in aqueous solution using gas bubbles (Matis et al. 1994). It is an effective method to harvest microalgae by taking advantage of their natural characteristics of relatively low density and self-float (Phoochinda and White 2003). Also with relatively rapid operation, low space requirements, high flexibility and moderate operational costs, flotation technique has the potential to overcome the bottleneck of feasible microalgal biofuel production (Liu et al. 1999). At present, there are mainly three flotation techniques reported for microalgae harvesting: dispersed air flotation (DiAF, bubble diameter 700–1500 µm), dissolved air flotation (DAF, bubble diameter 10–100 µm) and electrolytic flotation (Chisti, 2007, Phoochinda et al., 2004 and Uduman et al., 2010). Among these techniques, DiAF has been widely used to upgrade coal and minerals at large scale (cell volumes reaching up to 500 m³). DiAF seems to be an economical and efficient technique for harvesting
microalgae. At present, algae harvesting by flotation technique has only been developed for freshwater microalgae, such as *Chlorella vulgaris* and *Desmodesmus quadricauda* (Chen et al., 1998; Liu et al., 1999; Phoochinda and White, 2003 and Phoochinda et al., 2004). Although these studies inferred that the flotation efficiency could be affected by salinity (Liu et al., 1999 and Phoochinda and White, 2003), the flotation of marine microalgae has not been reported yet. In this study, the effectiveness of flotation on marine microalgae harvesting was investigated, through which hydrophobicity as a critical factor and a missing link between flotation performance and algal surface properties was identified.

**Methods**

**Cultivation of algae**

Marine microalga *Tetraselmis* sp. M8 was isolated from the Sunshine Coast, Queensland, Australia (26°39′39″S, 153°6′18″E; Genbank accession number JQ423158) and freshwater microalga *Chlorella* sp. BR2 was isolated from the Brisbane River, Tennyson, Queensland, Australia (−27°31′21.36″S, 153°0′32.87″E; Genbank accession number JQ423156; Lim et al., 2012). They were cultivated in silicate free f/2 medium, under 120 μmol photon m⁻² s⁻¹ with 12-h light/dark cycles, at 26 °C ± 1 °C on an orbital shaker (100 rpm). The cultivation was scaled up in two of 14 L cylindrical photobioreactors (one for each) with continuous supply of air and nutrients. When microalgae reached the exponential growth phase, they were nutrient-starved for two days for efficient lipid induction (Hu et al., 2008) and then collected for flotation experiments.

**Dispersed air flotation test**

Flotation experiments were carried out using a 1.5-L agitair flotation cell. Air was supplied to the flotation cell through its bottom, where an impeller was placed to provide the agitation necessary for breaking air into bubbles and dispersing them throughout the cell. The bubbles picked up microalgae and rose to the top, forming a microalgae-laden froth, which was subsequently removed manually. Prior to the flotation process, microalgae cultures were stirred vigorously for 2 min. Then each culture was subdivided into aliquots of 1.3 L, weighed and transferred into the flotation cell. The pH of the flotation pulp was adjusted to 9.5 by adding NaOH before adding the collector, tetradecyl trimethylammonium bromide (C₁₄TAB, molecular formula CH₃(CH₂)₁₃N(CH₃)₃(Br)). In the flotation cell, the microalgae suspension was first agitated by stirring at 800 rpm for 5 min.
Subsequently, the stirring speed was reduced to 600 rpm and aeration was turned on at a rate of 5 L min\(^{-1}\) (superficial air velocity 0.68 cm/s). Four concentrates were sequentially collected at 1, 2, 4, and 6 min. The cell count for each sample was taken in three duplicates by loading 10 μL of sample on a haemocytometer (Brightline, USA), and the average value was reported. The microalgae recovery (Y) and water rejection rate (WRR) were determined using Eqs. (1) and (2).

\[
Y = 1 - \frac{S_S}{F_f}
\]
\[
WRR = \frac{S}{F}
\]

Where \(S\) is the mass of sink (or tailing left in the flotation cell), \(F\) is the mass of feed, \(s\) is the microalgae concentration in the sink, and \(f\) is the microalgae concentration in the feed.

**Hydrophobicity test**

The hydrophobicity of microalgae was measured by using the modified adherence-to-hydrocarbon method (Rosenberg et al. 1980). The test assesses essentially the distribution ratio of cells between water and an organic phase. A total of 4 mL of the algae sample was placed in a test tube to which 1 mL of 98% pure n-hexane was added and shaken vigorously by hand for 1 min; the emulsion was allowed to settle for 2 min. Then, 2 mL were carefully obtained from the bottom aqueous layer of the test tube and its absorbance was read at 620 nm using a spectrophotometer (Hitachi, Model U-2800) to represent the concentration of microalgae. The extractability (\(H\)) of the hexane layer on organic substances in the algal suspension was calculated using the following expression:

\[
H = \left(\frac{A_o - A_w}{A_o}\right) \times 100\%
\]

Where \(A_o\) is the initial absorbance of the microalgae suspension and \(A_w\) is the absorbance of the aqueous phase after being settled for 2 min.

**Results and discussion**

**Changing collector dosage**

The flotation kinetics of freshwater microalga *Chlorella* sp. (BR2) in freshwater medium and marine microalga *Tetraselmis* sp. (M8) in seawater medium in the absence of any
collector were quite distinct. It was observed that within six minutes, 93% of BR2 could be recovered, whereas only 6% of M8 was recovered. It was hypothesized that M8 had a lower level of natural hydrophobicity than BR2 and that appropriate collectors were needed to render microalgae particles more hydrophobic. Most microalgae are negatively charged at natural pH values (Chen et al., 1998 and Phoochinda et al., 2004). Hence, in the present work, a cationic collector, tetradecyl trimethylammonium bromide (C$_{14}$TAB) was used for subsequent flotation experiments.

At a given flotation time, increasing collector dosage clearly increased the flotation recovery (Fig. 1). The addition of C$_{14}$TAB increased BR2 recovery to almost 99%, resulting in 30–40% more algae recovery in the first two minutes of flotation (Fig. 1a). A pronounced increase in microalgae recovery was seen when the C$_{14}$TAB concentration was increased from 1 to 3 ppm. However, there was no further improvement in the recovery when the C$_{14}$TAB concentration was further increased. The experimental data of cumulative flotation recovery versus flotation time were fitted by using the first-order chemical reaction analogy:

$$Y = Y_{max}(1 - e^{-kt}) \quad \text{equation (4)}$$

Where $Y_{max}$ is the maximum flotation recovery when the flotation time $t$ approaches infinity, and $k$ is the flotation rate constant. The coefficient of determination ($R^2$) of each curve fit was above 0.965, suggesting that the kinetics of the microalgae flotation process can be satisfactorily modelled by using Eq. (4). The value of $k$ increased considerably with increasing collector dosage from 0 to 3 ppm. Yet further increasing collector dosage to 10 ppm found little change in $k$. 
When considering water rejection for BR2, it was observed that within 6 min, 93% water could be removed with only 4.3% of algae cell loss. In other words, the concentration of algae cells could be increased by more than 10 times without significant loss of algae. This confirms that dispersed air flotation is effective for harvesting of freshwater algae.

When the concentration of C_{14}TAB was increased for marine microalga M8, there was a gradual increase in the recovery (Fig. 1b). It increased from 6% to 80% when C_{14}TAB was added from 0 to 80 ppm, suggesting that increasing collector dosage is beneficial for improving marine microalgal flotation recovery. On the other hand, increasing the collector dosage brought about lower water rejection rates. When the C_{14}TAB concentration was at 80 ppm, the water rejection rate was reduced to 60%, associated with overly stable froth.
At a given C\textsubscript{14}TAB concentration, the flotation recovery of M8 using seawater was much lower than that of BR2 using freshwater. Chen et al. (1998) observed that increasing the ionic strength reduced flotation recovery of microalgae. One could speculate that the relatively large ionic strength of seawater used for M8 flotation may have worsened the bubble-particle attachment. Ducker et al. (1994) found that hydrophobic force is the primary force for attachment of hydrophobic particles to air bubbles and the hydrophobic attraction force could be enhanced by increasing the hydrophobicity of particles. Therefore, a series of studies were performed to elucidate the role of microalgae hydrophobicity in microalgae flotation at various ionic strengths.

**Modifying salinity**

BR2 was cultivated as usual using freshwater but then 35 ppt NaCl was added and algae were incubated for various times before flotation commenced (Fig. 2a). The control experiment for BR2 flotation was performed without the addition of NaCl and subsequent incubation. The flotation recovery of BR2 in freshwater was 95.1%, and the measured hydrophobicity was 5.3%. When adding 35 ppt NaCl without subsequent incubation, the flotation recovery of BR2 was 97.1% and the measured hydrophobicity was 6.5%. Increasing the incubation time from 0 to 40 min and 22 h (with gentle agitation to avoid settling) had little effect on the flotation recovery and microalgae hydrophobicity. It is clear that ionic strength had little impact on the flotation response of BR2 and its hydrophobicity.
Figure 2. Flotation recovery and surface hydrophobicity of (a) freshwater microalgae BR2 with 3 ppm C14TAB at various NaCl concentrations and incubation times and (b) marine microalgae M8 at various C14TAB concentrations (3, 30, and 80 ppm) in freshwater and seawater. Each flotation test lasted for six minutes, and the overall flotation recovery was reported.

Comparative flotation tests for M8 were performed at three different concentrations of C14TAB in freshwater and seawater (Fig. 2b). M8 was cultivated as usual using seawater. The flotation tests in freshwater were carried out after centrifugation of M8 culture and redispersion of the microalgae in freshwater. The difference in flotation recovery of M8 between freshwater and seawater was small (less than 10 percentage points). Likewise, there was little difference between hydrophobicity of M8 in freshwater and seawater.
Hence, ionic strength played a rather minor role in determining the flotation of M8 and its hydrophobicity.

It is noteworthy that both flotation recovery and microalgae hydrophobicity increased with increasing collector dosage (Fig. 2b). When seawater was used in flotation, the correlation coefficients between flotation recovery and hydrophobicity were 0.84; when freshwater was used in flotation, the correlation coefficient was 0.85. By comparing the hydrophobicity of BR2 and M8 at 3 ppm collector (Fig. 2), one can see that M8 has a lower hydrophobicity (1.3%) than BR2 (5.3%), which corroborates the much poorer flotation performance of marine microalga M8 at 3 ppm C_{14}TAB. The good correlation between microalgae flotation recovery and hydrophobicity suggests that one can increase the flotation performance of microalgae by using more effective collectors which can render microalgae surface more hydrophobic.

Conclusions

If no collector was used, the flotation performance of marine microalga M8 was poorer than that of freshwater microalga BR2 because the former had a lower degree of hydrophobicity. The use of cationic surfactant C_{14}TAB enhanced M8’s hydrophobicity and thus its flotation recovery to a satisfactory level but the water rejection rate became low. In contrast, C_{14}TAB was an effective collector for the flotation of BR2 as its flotation recovery and water rejection rate were both high. The ionic strength of flotation medium had little influence on the flotation of M8 and BR2.

Acknowledgements

Financial support for this study, provided by a Linkage Grant (LP0990558) from the Australian Research Council and a CIEF grant by The University of Queensland and Queensland Sea Scallops Trading Pty Ltd, is gratefully acknowledged.

References


4. EFFECTIVE HARVESTING OF LOW SURFACE-HYDROPHOBICITY MICROALGAE BY FROTH FLOTATION

Background

In the previous chapter, hydrophobicity was identified as a critical factor and a missing link between flotation performance and algal surface properties. It was also identified that ionic strength does not have much effect on flotation. Thus, the important criterion for successful flotation is increasing the hydrophobicity of microalgae so that they can adhere to hydrophobic air bubbles. Flotation performance can be affected by chemical and hydrodynamic factors.

Among the variables that affect the chemical condition of froth flotation, pH, surfactant type and concentration play critically important roles in affecting the electrical charge and hydrophobicity of the microalgal particle surface. At the same time, various types of flotation devices which provide different hydrodynamic conditions may also affect flotation separation performance. For example, smaller bubbles generated by using different types of flotation machines can improve fine particle flotation.

Hence a step-wise comparative study was required to understand the effects of different surfactants, pH, cell concentration, and machines on microalgae flotation efficiency with low and high hydrophobicity microalgae.

The results for this study were published as part of a paper entitled: Effective harvesting of low surface-hydrophobicity microalgae by froth flotation. Published in Bioresource Technology.

Key findings:

- Algal hydrophobicity and bubble size are key factors for microalgae flotation.
- Algal hydrophobicity can be improved using cationic surfactants at appropriate pH levels.
- A step-wise optimisation of algae flotation is demonstrated.
EFFECTIVE HARVESTING OF LOW SURFACE-HYDROPHOBICITY MICROALGAE BY FROTH FLOTATION

Sourabh Garg\textsuperscript{a}, Liguang Wang\textsuperscript{b}, Peer M. Schenk\textsuperscript{a}

\textsuperscript{a} Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane QLD 4072, Australia
\textsuperscript{b} School of Chemical Engineering, The University of Queensland, Brisbane QLD 4072, Australia

Received 29 January 2014, Revised 4 March 2014, Accepted 6 March 2014, Available online 15 March 2014

Abstract

Microalgae harvesting by air flotation is a promising technology for large-scale production of biofuel, feed and nutraceuticals from algae. With an adherence-to-hydrocarbon method and two different types of flotation cells (mechanically agitated cell and Jameson cell), microalgal surface hydrophobicity and bubble size were identified to be critical for effective froth flotation of microalgae. Freshwater alga \textit{Chlorella} sp. BR2 showed naturally a high hydrophobicity and an ideal response to flotation. However, many marine microalgae possess a low surface hydrophobicity and are thus difficult to harvest. This paper shows that a step-wise optimization approach can substantially improve the flotation of a low surface hydrophobicity marine microalga, \textit{Tetraselmis} sp. M8, to near full recovery with an enrichment ratio of 11.4.

Highlights

- Algal hydrophobicity and bubble size are key factors for microalgae flotation.
- Algal hydrophobicity can be improved using cationic surfactants at appropriate pHs.
- A step-wise optimization of algae flotation is demonstrated.

Keywords

Marine microalgae; Froth flotation; Hydrophobicity; Cationic surfactant; Bubble size
Introduction

Microalgae are considered the most efficient primary producers of biomass. They have great potential to be a future feedstock for producing biofuel and other products as their cultivation does not need to compete for arable land or biodiverse landscapes. Many marine microalgae can use brackish or seawater and are highly efficient producers of lipids. The industrial production of biofuel from microalgae can be divided into three major steps; cultivation, harvesting and processing (Ryan, 2009). Among these, one of the major impediments for commercial-scale production is the downstream processing, where algal biomass has to be concentrated and separated (dewatered) from water for further processing (Christenson and Sims, 2011 and Molina Grima et al., 2003). This step can contribute to 20-30% of total biofuel production costs (Molina Grima et al., 2003). Commercial production of microalgal biodiesel requires efficient harvesting and dewatering of algal biomass (Cheng et al., 2010). Various procedures such as flocculation, sedimentation, filtration, flotation, centrifugation and membrane separation have been established for primary dewatering of microalgae from the cultivation medium (Phoochinda and White, 2003). However, each approach has its own limitation; typically, they are either of low efficiency or high capital cost with excessive energy consumption or cannot be applied at large scale.

Froth flotation presents a promising approach for commercial-scale harvesting of microalgae that compared to other methods, is also relatively low cost (Sharma et al., 2013). It utilizes microalgae’s natural features of relatively low density and self-float (Phoochinda and White, 2003) and is considered a highly versatile method for physically separating particles with a small footprint (Chen et al., 1998 and Garg et al., 2012). Microalgal cells are small particles whose size typically ranges from 1 to 20 micron. A missing link between flotation performance and algal surface hydrophobicity has recently been identified and algal hydrophobicity has now been recognized as a major factor determining microalgae flotation efficiency, irrespective of whether these are marine or freshwater microalgae (Garg et al., 2012). Addition of surfactants is commonly used to render algae surface hydrophobic, making it possible to use surfactants as carriers for flotation to separate microalgae from water (Chen et al., 1998, Garg et al., 2012 and Uduman et al., 2010). Flotation efficiency can be affected by hydrodynamic and chemical factors. Variables that affect the chemical condition of froth flotation include pH, surfactant type and concentration, as these play important roles affecting the
hydrophobicity and electrical charge of particle surfaces (Bulatovic, 2007). Various types of flotation devices which provide different hydrodynamic conditions may also affect flotation separation performance. For example, smaller bubbles generated by using different types of flotation machines can improve fine particle flotation (Yoon, 2000 and Zhou et al., 1997). The Jameson Cell is an advanced flotation apparatus that employs a plunging jet to produce smaller air bubbles than mechanical flotation cells. The Jameson Cell technology was originally applied by Yan and Jameson to treat wastewater (Yan and Jameson, 2004), with microbial removal efficiencies over 98% (on the basis of the difference in concentration between feed and tail).

In the present work, a step-wise comparative study was carried out to understand the effects of different surfactants, pH, cell concentration, and machines on microalgae flotation efficiency with low and high hydrophobicity microalgae. Microalgal recovery for marine microalgae *Tetraselmis* sp. M8 was improved from an initial 6.4% to 97.4% with a satisfactory enrichment ratio of 11.4. Microalgal surface hydrophobicity and bubble size were identified as the main underlying causes that improved froth flotation performance.

**Methods**

**Algal culture and characterization**

Pure cultures of the green marine microalga *Tetraselmis* sp. M8 were obtained from a coastal rock pool in Maroochydore, Queensland, Australia (26° 39’ 39” S, 153° 6’ 18” E; Genbank accession number JQ423158) and the green freshwater microalga *Chlorella* sp. BR2 was isolated from the Brisbane River, Tennyson, Queensland, Australia (27° 31’ 21.36” S, 153° 0’ 32.87” E; Genbank accession number JQ423156) (Lim et al., 2012). Microalgae stocks are maintained in the Algae Biotechnology Laboratory at The University of Queensland, Australia (www.algaebiotech.org). Cultures were grown in silicate free f/2 medium, on an orbital shaker (100 rpm) at 26 °C ± 1 °C under 120 μ mol photon m⁻² s⁻¹ with 12-h light/dark cycles. Using the same conditions, cultures were scaled up in two 20 L polyethylene bags with daily nutrient and continuous air supplies. When microalgal cultures reached the end of the exponential growth phase (less than 20% increase in cell numbers per day), they were nutrient-starved for 2 d for lipid induction (Hu et al., 2008). Subsequently microalgal cultures were used for flotation experiments.
Froth flotation

Flotation experiments were carried out using a 1.5-L bottom-driven mechanically agitated (Agitair) cell, unless otherwise stated. Microalgal cultures were stirred vigorously for 2 min, before each culture was subdivided into aliquots of 1.3 L, weighed and transferred into the flotation cell. The pH of the flotation pulp was adjusted with HCl or NaOH before adding the collector, tetradecyl trimethyl ammonium bromide (C\textsubscript{14}TAB) or dodecyl ammonium hydrochloride (DAH). First the microalgal suspension was conditioned by mixing at 800 rpm for 5 min. The agitation rate was 600 rpm or 800 rpm when C\textsubscript{14}TAB or DAH was used for flotation tests, respectively and the air flow rate was 5 L/min. The mechanical flotation lasted for 6 min.

Once an optimal reagent scheme was determined by the above-described mechanical flotation tests, additional Jameson cell flotation tests were carried out to determine the effect of bubble size (or flotation hydrodynamics) on microalgae flotation. The diameter size of the Jameson Cell used was 150 mm and its orifice diameter was 3.83 mm. A 35-L slurry was fed into the Jameson Cell at a pressure of 150 kPa and an air flow rate of 10 L/min. The Jameson cell flotation time was around 15 min. During this procedure the tailing was continuously recycled to the feed sump and pumped back to the Jameson cell. The Jameson Cell flotation procedure has previously been well described (Bulatovic, 2007 and Yan and Jameson, 2004). Microalgae cell count and dry weights were determined for concentrates collected in trays and remaining tailings left in the flotation machine. Triplicate cell counts were carried out for each sample by loading 10 \( \mu \)L of sample on a haemocytometer (Brightline, USA), and the averaged value was determined. Microalgae recovery (\( Y \)) was determined using the following equation:

\[
Y = 1 - \frac{T_t}{F_f}
\]  

Equation (1)

where, \( T \) is the wet mass of tailing (or sink), \( F \) is the wet mass of feed, \( t \) is the microalgal concentration in the tailing, and \( f \) is the microalgal concentration in the feed.

The enrichment ratio (\( ER \)) was calculated as the ratio of the concentration of algae in the concentrate to the concentration of algae in the feed. The following formula was used:

\[
ER = \frac{Y}{1 - WRR}
\]  

Equation (2)
Where $WRR$ represents the water rejection rate as equal to $T/F$.

**Hydrophobicity test**

Hydrophobicity ($H$) of microalgae was quantified by employing a modified adherence-to-hydrocarbon method (Rosenberg et al., 1980). We followed the same procedure as described by Garg et al., 2012 except that the emulsion was allowed to settle for only 20 s.

**Results and Discussion**

An initial comparison of the freshwater microalga *Chlorella* sp. BR2 with the marine microalga *Tetraselmis* sp. M8 showed that at pH 9.5, BR2 possessed much higher natural surface hydrophobicity than M8. The flotation recovery of *Chlorella* sp. BR2 reached more than 90% with a satisfactory enrichment ratio of 13.5, while, interestingly, only 6.4% recovery with an enrichment ratio of only 0.6 was measured for *Tetraselmis* sp. M8 under identical process conditions. Note that the enrichment ratio of M8 flotation was less than 1, which was most likely caused by the (downward) gravitational sedimentation, which counteracted the (upward) flotation of microalgae.

The baseline flotation response of *Tetraselmis* sp. M8 in the absence of any modifications was poor, but was considered an ideal model to determine the factors how froth flotation for a low performing microalga can be improved. First, appropriate surfactants can be used as flotation collectors which can render the surface of microalgal cellular structures more hydrophobic. According to published data, most microalgae are negatively charged at a neutral pH, so cationic surfactants are frequently used as flotation collectors for microalgae (Chen et al., 1998 and Phoochinda et al., 2004). In the present work, as a first attempt, a range of flotation tests were carried out with C$_{14}$TAB as a collector. At various concentrations, the influence of C$_{14}$TAB on algal surface hydrophobicity and flotation performance was systematically analyzed. As shown in Fig.1, addition of C$_{14}$TAB considerably increased the cellular surface hydrophobicity and flotation response of *Tetraselmis* sp. M8. As C$_{14}$TAB concentration increased from 0 to 80 ppm, algal hydrophobicity increased from 1.2% to 25.0%, the flotation recovery was improved from 6.4% to 81.7%, and the enrichment ratio peaked at 3.4 at a dosage of 50 ppm. While higher C$_{14}$TAB concentrations considerably improved flotation recovery, it also caused an increased amount of water to be transported to the flotation concentration stream, hence less water was rejected and a lower enrichment ratio was measured. This observation can be explained by the slower liquid drainage in a saline water film confined between air
bubbles at higher concentrations of flotation reagents (Wang, 2012). In the present study, the flotation froth became overly stable when increasing C_{14}TAB dosage to high levels. Ideally, a metastable froth is desirable for accomplishing both, a high microalgae recovery and a high enrichment ratio.

In seeking for more effective collectors for dewatering of low hydrophobicity marine microalgae, the hydrophobicity of Tetraselmis sp. M8 in aqueous solutions of DAH was first measured. The cultures were also adjusted to different DAH concentrations and pH values. As shown in Fig 1a, DAH was more capable of increasing M8’s hydrophobicity than C_{14}TAB. Varying DAH dosage from 0 to 50 ppm gradually increased the algal hydrophobicity. Furthermore, at a given DAH concentration, pH 6 resulted in higher hydrophobicity compared to pH 4 and 9.5. The highest algal hydrophobicity (>50%) was measured at 25 ppm DAH at pH 6 or at 50 ppm DAH at pHs 4 – 9.5.

Consistent with increased hydrophobicity, higher dosages of DAH also improved the flotation recovery (Fig 1b). Furthermore, pH had a strong influence on Tetraselmis sp. M8 flotation. At a set DAH concentration, the flotation recovery was always higher at pH 6 than at pH 4 or 9.5. Similar to C_{14}TAB, an increasing amount of DAH collector first augmented the enrichment ratio at 10 ppm before decreasing at higher concentrations (Fig 1c). The lower enrichment ratio at higher DAH concentrations was caused by the decreased water
rejection rate. In particular, at pH 6, when DAH dosage was raised from 10 ppm to 50 ppm, the water rejection ratio was clearly reduced from 93.0% to 68.7%. A pH of 6 resulted in the best overall flotation performance.

However, over the tested DAH concentration range, one could not concurrently measure the highest flotation recovery and the highest enrichment ratio. Hence flotation experimental conditions of 25 ppm DAH and pH 6 were selected as a compromise, where the flotation recovery was 85.0% with an enrichment ratio of 5.6. This assay was then repeated twice under these conditions, and the data were then directly compared with the best flotation performance results for C\textsubscript{14}TAB experiments that were repeated once. As shown in Fig. 2a, using DAH as a collector resulted in a higher flotation recovery and larger enrichment ratio than C\textsubscript{14}TAB. It is particularly noteworthy that DAH improved the enrichment ratio to 5.8 with 86.8% algae recovery. By comparison, the enrichment ratio was 3.4 with a flotation recovery of 71.1% when using 50 ppm C\textsubscript{14}TAB or 2.0 at 80 ppm C\textsubscript{14}TAB with a flotation recovery of 81.7%.
Figure 2 (a) Direct comparison of DAH and C_{14}TAB as collector for mechanical flotation performance of *Tetraselmis* sp. M8; (b) Enrichment ratio versus flotation recovery for microalgal cultures *Tetraselmis* sp. M8 using mechanical cell and Jameson cell with 25 ppm DAH at pH 6. Lines are drawn to guide the eye, and are expected to follow towards a recovery limitation of 100% at which the enrichment ratio would be 1.

To determine whether devices considered to be more efficient and capable of generating smaller bubbles may further improve flotation performance, three flotation assays were carried out with a Jameson flotation cell. Conditions included the use of 25 ppm DAH as a collector and a pH-adjusted culture at pH 6 (Fig.2b). To allow a direct comparison between both flotation cells, data collected under the same chemical conditions are included for the mechanical cell. As shown in Fig.2b, the Jameson cell resulted in a superior M8 enrichment ratio of 11.4 and a recovery rate 97.4%. The use of a different flotation machine led to a near doubling of the enrichment ratio and even the flotation recovery increased by at least 10%. For example at a given flotation recovery of 86%, the Jameson cell generated a remarkably higher enrichment ratio (25.9), which was 4.5 times higher than that with the mechanical cell. The desirable flotation performance with the Jameson cell can be attributed to the smaller bubbles (less than 500 micron). The average of bubble size in conventional industrial flotation operations is 1–2 mm. Use of small bubbles can considerably improve fine particle flotation (Yoon, 2000 and Zhou et al., 1997). Smaller bubbles can interact with mineral particles at higher collision probability, higher attachment probability and lower detachment probability, lower ascending rate and higher free surface energy, which are all desirable for flotation. However, other properties of the Jameson cell may also contribute. For example, the Jameson cell has intense mixing with small bubbles, tailings recycling, no mechanical agitation and no external air supply. Further research should be carried out to better understand the flotation performance of marine microalgae using the Jameson Cell technology and whether this can be applied at large industrial scales.

To determine dependency of flotation recovery and algal surface hydrophobicity, both parameters were plotted in Fig.3. It shows that when C_{14}TAB or DAH was used as the collector, a strong correlation exists between flotation recovery and algal hydrophobicity. Although C_{14}TAB could be used to improve the hydrophobicity of the marine microalga *Tetraselmis* sp. M8, DAH was much more effective for rendering the surface of this microalga hydrophobic. Typically, a higher packing density of the collector hydrocarbon tails on the particle surface should result in a stronger surface hydrophobicity. A collector that can be better adsorbed onto microalgal cellular surfaces may also enable the formation of microalgae aggregate, which is favourable for improving fine particle
flotation. Xu and Yoon, (1990) showed that hydrophobic coagulation, which is driven by the hydrophobic force, also plays an important role in flotation, and the kinetics of coagulation increase with increasing particle hydrophobicity.

Figure 3 Flotation recovery ($Y$) as a function of algal surface hydrophobicity ($H$) for M8 with using (a) C$_{14}$TAB and (b) DAH. The straight line represents the best linear fit to all the data points shown in this subset.

The observed correlation between microalgae flotation recovery and hydrophobicity suggests that considerable increases in flotation performance of microalgae can be expected by using more effective collectors that can increase the hydrophobicity of microalgal surfaces. Quantification of hydrophobicity for microalgae presents a simple and effective assay that allows screening of flotation collectors and other chemical conditions of microalgal cultures, prior to using elaborate froth flotation assays. Results from the present study demonstrate that even low hydrophobicity marine microalgae can be harvested by froth flotation. Culture properties, chemical reagents and bubble size (hydrodynamics) are important for the flotation of marine microalgae. Further studies need to determine whether collectors can possibly interfere with downstream processing (including water recycling) and whether application of froth flotation for microalgae harvesting can help establish large-scale commercial cultivation.

Conclusions

Microalgae with low surface hydrophobicity are difficult to harvest by flotation separation. C$_{14}$TAB as collector improved the flotation recovery of marine microalga, Tetraselmis sp. M8 in a mechanically-agitated cell from 6.4% to 81.7%, but with an unsatisfactory enrichment ratio of 2.0. DAH rendered cells more hydrophobic, improving the enrichment
ratio to 5.8 with 86.8% recovery. A Jameson cell with relatively small bubbles resulted in an enrichment ratio of 11.4 with 97.4% recovery. It is concluded that surface hydrophobicity and bubble size are key factors affecting algae flotation, and a step-wise optimization can lead to effective flotation separation of difficult-to-harvest microalgae.

Acknowledgements

Financial support for this study, provided by the Australian Research Council and a CIEF grant by The University of Queensland, is gratefully acknowledged.

References


5. Flotation Separation of Marine Microalgae from Aqueous Medium

Background

In the previous study it was concluded that surface hydrophobicity and bubble size are key factors affecting algae flotation. In addition, a step-wise optimisation can lead to effective flotation separation of difficult-to-harvest microalgae. However variation in collector’s performance at different pH suggested that:

- Collectors behave differently at different pH and can provide different levels of hydrophobicity to microalgae; or that,
- Microalgae behave differently at different pH and can have different levels of hydrophobicity causing variation in flotation performance.

Hence there was a need to identify the effect of pH on collector and microalgal hydrophobicity and to determine the methods to improve flotation at higher pH levels. Another important parameter that should be optimised is the water recovery. The amount of water recovered mainly depends upon the froth stability. In the field of flotation, only meta-stable froth can result in good water rejection. Hence there is a need to study the froth stability of different collectors as well as elucidate the influence of the surfactant head group on the flotation efficiency of marine microalgae at various pH levels.

The result for this study will be published as part of a paper entitled: Improving microalgae flotation via adjusting collector chemistry and hydrodynamics. Paper ready for submission,

Key findings:

- Surfactant precipitation is detrimental to microalgae flotation.
- By selecting the appropriate collector for harvesting microalgae at higher pH, algae recovery can be improved and can be further made cost-effective.
- Use of DPC and a Jameson Cell led to a 23-fold increase in algae concentration, with over 99% of marine microalgae recovered.
- Higher froth stability leads to a higher water recovery and thus a lower enrichment ratio.
Sourabh Garg\textsuperscript{a}, Liguang Wang\textsuperscript{b,*}, Peer M. Schenk\textsuperscript{a,*}

\textsuperscript{a} Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane QLD 4072, Australia.
\textsuperscript{b} School of Chemical Engineering, The University of Queensland, Brisbane QLD 4072, Australia.

Abstract

Harvesting of oleaginous marine microalgae is an important step for cost-effective algal biomass feedstock production. This study reports separation of marine microalgae (\textit{Tetraselmis} sp. M8) from aqueous medium by froth flotation using various collectors (surfactants) with equal carbon chain length, such as dodecyl pyridinium chloride (DPC), N-dodecylpropane-1,3-diamine hydrochloride (DN2), dodecyl amine hydrochloride (DAH), and sodium dodecyl sulphate (SDS), at different pHs. Algal hydrophobicity, froth stability, and surfactant precipitation were characterised. The mechanical flotation tests show that at natural pH 9.5 and a lower pH, DPC outperformed DAH, DN2 and SDS in separating M8 from seawater. DPC was able to render the microalgae hydrophobic and produce metastable froth, and had relatively high solubility in water, which are all desirable features of a collector for microalgae flotation. At pilot scale outdoor cultivation using DPC for a Jameson cell flotation led to a 23-fold increase in algal concentration with over 99% algal recovery.

\textit{Keywords}: Marine microalgae; froth flotation; hydrophobicity; surfactant solubility; bubble size.

* Corresponding authors:
Tel: +61 7 3365 7942; e-mail address: Liguang.Wang@uq.edu.au (L. Wang);
Tel: +61 7 3365 8817; e-mail address: p.schenk1@uq.edu.au (P. Schenk).

Introduction

Substituting the use of fossil fuel with biofuel that is more sustainably sourced can minimise the carbon footprint (Brennan and Owende 2010). Microalgae are highly productive photosynthetic microorganisms that can provide biofuel independent of using fresh water or arable land (Chisti 2007; Christenson and Sims 2011; Schenk et al. 2008; Sheehan et al. 1998). The overall production of biofuel from microalgae can be divided into three major steps: algae cultivation, harvesting and processing (Ryan 2009). Among these steps, the major bottleneck for commercial-scale production of biofuels is the harvesting
This step can consume 20 to 30% of total biofuel production costs (Molina Grima et al. 2003; Phoochinda and White 2003). Mass production of microalgal biodiesel requires efficient harvesting of the biomass from cultivation media (Cheng et al. 2010).

Based on previous studies, flotation technique is one of the most promising methods for commercial-scale dewatering of microalgae and concentrating the algal biomass from approx. 0.05-0.1% to 1-3% by weight (Garg et al. 2014). Flotation is a highly versatile method for physically separating the suspended particles in liquid (Chen et al. 1998; Garg et al. 2012). It consists of three phases: water, solid particles and air bubbles. During the initial stages of collision owing to the deformation of bubbles, a thin liquid film referred to wetting film is formed between the bubble and the particle (Pan et al. 2012). Destabilisation of this film results in adhesion of hydrophobic particle to the air bubble, which is the basis for froth flotation (Farrokhpay 2011; Pan et al. 2012; Perea-Carpio et al. 1988). An important factor for microalgae flotation is the hydrophobicity of microalgal cells (Garg et al. 2014). The surfaces of microalgae vary from being naturally hydrophilic to slightly hydrophobic. Addition of surfactants (collector) can effectively render microalgal surfaces more hydrophobic, making it easier to separate algae from water (Chen et al. 1998; Garg et al. 2012; Liu et al. 1999; Uduman et al. 2010). A collector consists of a hydrophilic head and a hydrophobic carbon tail, and its adsorption onto microalgae helps these microorganisms to adhere to air bubbles after which they are transported to the froth zone (Garg et al. 2014). It is the charged head of a surfactant that binds to microalgae. It is speculated that the electrostatic interactions between the collector and the particles (in this case microalgae) plays a key role in the efficiency of the collectors. Since electrostatically charged collectors are used; they have polar head. As the surface of most algae is negative, cationic surfactants are mostly used (Perea-Carpio et al. 1988). Moreover different head group will perform differently under similar testing conditions. Collector adsorption can be affected by multiple factors such as concentration and pH. With operating costs taken into consideration, low surfactant dosage and natural pH should be employed wherever possible.

pH plays an important role in affecting collector adsorption onto particles and thus flotation performance. At alkaline pHs, microalgae are often negatively charged; reducing pH could make the microalgal surface charge neutral or even positively charged. For a cationic surfactant or anionic surfactant, there should be an optimum pH range at which one can take advantage of the strong electrostatic interaction between microalgae and surfactant to
hydrophobize the surface of microalgae (Chen et al. 1998; Liu et al. 1999). Various studies demonstrate that cationic collectors tend to have better performance in slightly alkaline pH whereas anionic collectors work better when the pH of suspensions becomes acidic (Chen et al. 1998; Liu et al. 1999; Phoochinda and White 2003). pH modification can lead to various changes, including changes in interfacial chemistry (including surface charges), solubility of particles such as collectors, and water chemistry (Fuerstenau et al. 2007).

Air bubbles generated in flotation not only provides a platform for hydrophobic microalgae to attach but also enhance particle recovery by providing the lifting force required for transportation and separation. Flotation separation efficiency is inversely related to bubble size (Dai et al. 1998; Dai et al. 2000). Modern flotation technology utilizes efficient ways of enabling bubble–particle interactions in the liquid medium. Effective flotation requires high bubble-particle collision, attachment and stability efficiencies before reaching the pulp-froth interface (Derjaguin and Dukhin 1993). Small bubbles have a higher surface area to volume ratio. One of the most efficient ways of achieving maximum attachment is by generating as many small bubbles as possible (Hanotu et al. 2012). The flotation separation performance of fine particles can be enhanced 100-fold with bubble size reduction by 10 times (Ahmed and Jameson 1985). Furthermore, small bubbles have low rise velocity (Schulze 1992) enabling them to remain in liquid medium for longer time thereby increasing the attachment efficiency.

In the present work, dispersed-air flotation was used to separate marine microalgae from water. The study was focused on elucidating the influence of the surfactant head group on the flotation efficiency of marine microalgae at various pHs. The surfactants include three cationic surfactants and one anionic surfactant, with equal carbon chain length. Important factors affecting microalgae flotation were identified, including the affinity of collectors to algal surface, the solubility of collectors, and froth stability.

**Materials and Methods**

The surfactants tested (Fig. A.1, Supplementary data) included dodecyl pyridinium chloride (DPC, 99% pure, Sigma Aldrich), sodium dodecyl sulphate (SDS, 98.5% pure, Sigma Aldrich), and dodecyl amine hydrochloride (DAH, 97% pure, Alfa Aesar), which were used without further purification, and N-dodecylpropane-1,3-diamine hydrochloride (DN$_2$) solution, which was prepared by dissolving N-dodecylpropane-1,3-diamine (97% pure, Nanjin Chemlin Chemical Industry) in hydrochloric acid at a 1:1 mole ratio. Artificial
sea salt was purchased from Aquasonic. f/2 medium was purchased from Algaboost. HCl was used to adjust the pH.

**Cultivation of algae**

The marine microalga *Tetraselmis* sp. M8 was isolated from the Sunshine Coast, Queensland, Australia (26°39'39"S, 153°6'18"E; Genbank accession number JQ423158) (Lim et al. 2012). Microalgae were cultivated in silicate free f/2 medium, under 120 µmol photon m⁻² s⁻¹ with 12-hour light/dark cycles, at 26°C ± 1°C on an orbital shaker (100 rpm). The cultivation was scaled up in two 25 L polyethylene bags with continuous supply of air and nutrients. When microalgae reached the exponential growth phase, they were nutrient-starved for two days for efficient lipid induction (Hu et al. 2008) and then collected for flotation experiments. The natural pH of the culture samples prior to flotation was 9.5.

**Hydrophobicity test**

The hydrophobicity of microalgae was measured by using the modified adherence-to-hydrocarbon method (Rosenberg et al. 1980). We followed the same procedure as described by Garg et al., 2014 except that 1 mL sample from the bottom aqueous layers of the test tube was obtained for absorbance measurement.

**Dispersed air flotation**

**Mechanical cell:** Flotation experiments were carried out using a 1.5-litre Agitair flotation cell. Air was supplied to the flotation cell through its bottom, where an impeller was placed to provide the agitation necessary for breaking air into bubbles and dispersing them throughout the cell. The bubbles picked up microalgae and rose to the top, forming a microalgae-laden froth, which was subsequently removed manually. Prior to the flotation process, microalgal cultures were stirred for 1 min. Then each culture was subdivided into aliquots of 1.3 litres, weighed and transferred into the flotation cell. The pH of the flotation pulp was adjusted by adding NaOH or HCl before adding the collector. In the flotation cell, the microalgae suspension was first agitated by stirring at 800 rpm for 5 min. After conditioning, aeration was turned on at a rate of 5 litre min⁻¹.

**Jameson cell:** A pilot-scale Ø150 mm Jameson cell test unit was used to perform further flotation tests to understand the effect of bubble size (or flotation hydrodynamics) on microalgae flotation. More details on this machine can be found elsewhere (Garg et al. 2014). Each Jameson flotation test requires 35-L slurry, which was fed into the Jameson
Cell at a pressure of 150 kPa and an air flow rate of 10 L/min. The Jameson cell flotation time was around 15 min.

The concentrates collected in trays and the tailings left in the flotation machine underwent weighing and microalgae cell counting. The cell count for each sample was taken in three duplicates by loading 10 µL of sample on a haemocytometer (Brightline, USA), and the averaged value was reported. The microalgal recovery ($Y$) and water recovery ($WR$) were determined using the following equation:

$$Y = 1 - \frac{T}{F}$$  \hspace{1cm} (1)

$$WR = 1 - \frac{t}{f}$$  \hspace{1cm} (2)

where $T$ is the mass of tail left in the flotation cell, $F$ is the mass of feed, $t$ is the microalgae concentration in the tail, and $f$ is the microalgae concentration in the feed.

The enrichment ratio ($ER$) is defined as the ratio of the concentration of algae in the concentrate to the concentration of algae in the feed. It was calculated using the following formula:

$$ER = \frac{Y}{WR}$$  \hspace{1cm} (3)

**Collector solubility**

The seawater was prepared using tap water mixed with artificial sea salts. The final salinity was adjusted to 35 ppt and 100 mL water each was poured into seven 200 mL beakers. The pH in each beaker was adjusted to the pH 6 to 10 with the increments of 0.5 pH using NaOH or HCl. From each beaker four replicates of 10 mL each were transferred into 20 mL test tubes. A total of 40 µL of 1% solution (DPC, DN2, DAH or SDS) was added to each test tube to set the final concentration of collector (i.e., 40 ppm). Tubes were then vortexed for 15 seconds and the optical density (OD) was read at 620 nm using a spectrophotometer (Hitachi, Model U-2800). The graph of OD at 620 nm vs. pH was plotted to infer the dependence of surfactant solubility on pH based upon the turbidity of solution.
Froth stability

A modified Bikerman test (foam rise) method was used for measuring froth stability in the same Agitair flotation cell as in the microalgae flotation tests, with a square (9 × 9 cm), vertical (40 cm), transparent (Perspex) column mounted onto the top of the flotation cell. The froth level in the column was measured visually and recorded as a function of time. After some time, depending on the froth stability and operating conditions, the froth reaches a constant height when there is no further growth. The maximum froth height at steady state with continuous bubbling was then recorded. The method can also give the froth decay time after the air supply is switched off.

Results and Discussion

In the absence of any collectors, the surface hydrophobicity and the overall recovery of marine microalgae *Tetraselmis* sp. M8 were low (Garg et al. 2012; Garg et al. 2014). To improve these values, one can use appropriate collectors to render the microalgal surface more hydrophobic. For the sake of minimising reagent cost, the pH at which the flotation is to be carried out should be at the natural value, wherever possible. In the present work, flotation tests for *Tetraselmis* sp M8 culture were first carried out at its natural pH (pH 9.5), then at a lower pH. Flotation performance was assessed by enrichment ratio versus algal recovery.

Flotation of microalgae at natural pH

Figure 1 shows the overall recovery and enrichment ratios of marine microalgae at natural pH (9.5) in the presence of four different collectors. At 10 ppm or 25 ppm, the four collectors showed significant differences in the flotation performance. DPC apparently outperformed the other three collectors in that the former gave the highest enrichment ratio and algal recovery. For a given collector, increasing the dosage from 10 ppm to 25 ppm generally recovered more microalgae and water to the product stream (Fig. A.2, Supplementary data). According to Eq. (3), the higher the water recovery, the lower the enrichment ratio is.
Flotation of microalgae at pH 6

To determine the effect of decrease in pH on the microalgal recovery, flotation tests were performed at pH 6.0 using the same collectors and concentrations that were used at pH 9.5. The results are shown in Figure 2. When the pH value was reduced from 9.5 to 6, we observed significantly increased microalgal recovery and slightly increased water recovery for DN2, DAH and SDS. As a result, the enrichment ratios for DN2, DAH and SDS were increased. However, the pH change resulted in nearly no change in the overall microalgal recovery and water recovery for DPC.
Figure 2. Microalgal recovery and enrichment ratio at pHs 6.0 and 9.5 with collectors at 10 ppm and 25 ppm, respectively. Error bars represent standard errors of mean values from three replicates. Data of DAH were adapted from Garg et al 2014.

Flotation recovery of microalgae is dependent on algal hydrophobicity.

In the present work, flotation recovery of microalgae was mainly related to collector chemistry, which in turn determines algal hydrophobicity. When plotting algal recovery against algal hydrophobicity, a linear relationship apparently exists (see Figure 3). The highest hydrophobicity was obtained at pH 6.0 with 25 ppm DN2, which is consistent with the highest yield achieved at that condition. The results obtained in the present work confirm our previous finding that algal hydrophobicity plays a decisive role in determining algal recovery (Garg et al. 2012).

Figure 3. Dependence of algal recovery (Y) on algal hydrophobicity (H). The straight line represents the best linear fit to all the data points. Data of DAH were adapted from Garg et al 2014.

For SDS, DAH and DN2, at the same concentration, one can see that changing pH leads to a change in algal hydrophobicity and algal recovery. More specifically, lower pH values
would lead to higher $H$ and $Y$. On the other hand, the recovery and hydrophobicity of microalgae with DPC as collector did not change with the change in pH.

**Higher froth stability leads to higher water recovery and thus a lower enrichment ratio**

From a perspective of increasing recovery and enrichment ratio, there is an optimal froth stability for any given flotation cell and operating conditions. The correct stability of the froth is very important as too stable froth is difficult to handle but, on the other hand, an unstable froth collapses rapidly (Farrokhpay, 2011). Ideally, the froth of microalgae flotation should be stable for short periods of time and breaks quickly once it is skimmed off from the system, resulting in minimum water recovery. Figure 4 shows a linear relationship between water recovery and froth stability. Among all the collectors in this study, DPC gave the lowest water recoveries and desirable froth stabilities by providing adequate froth height and rapid froth decay time. With a decrease in pH there was an increase in water recovery for DN2, DAH and SDS (see Figure 4).

![Figure 4. Dependence of water recovery (WR) on froth stability represented by the maximum froth height. The straight line represents the best linear fit to all data points.](image)

**Surfactant precipitation is detrimental to microalgae flotation**

The anionic surfactant SDS being negatively charged preferred a lower pH to render algal surface hydrophobic as in acidic pH algae would undergo charge reversal and therefore would be able to bind SDS. From Figure 2, one can see at pH 9.5 that $Y$ (DPC) was higher than $Y$ (DN2), whereas at pH 6, $Y$ (DPC) was lower than $Y$ DN2). To further understand the
effect of pH on collector performance, absorbance (A) of DAH, DN2 and DPC at 40 ppm in seawater at various pH values was measured at a wavelength of 620 nm. This collector concentration was the closest to the collector dosages used in the flotation tests while allowing the spectrometer to produce meaningful results. As shown in Figure 5, the absorbance for the DPC solution was the lowest, whereas the DAH solution gave the highest absorbance. The increasing absorbance signifies precipitation of collectors. It is suggested that at pH 9.5 there were nearly no surfactant crystals in the DPC solution whereas a significant amount of surfactant crystals were formed in the DN2 and DAH solutions. It was reported by others that when the pH exceeded 9, the solubility limit of DAH is very low (Dai & Laskowski, 1991). It was also observed that DPC was completely soluble in seawater at and below pH 9.5. SDS was soluble at and below pH 9, whereas DAH and DN2 were completely soluble at and below pH 7. By binding to the microalgal surface, collectors help to improve the microalgal hydrophobicity. Thus, resulting in an increase in the recovery performance. The precipitation of collectors at pH 9.5 would result in decreased availability of active collector for microalgae thereby, rendering surface less hydrophobic.

![Figure 5. Absorbance (at 620 nm) as a function of pH for different cationic collectors. The absorbance indicates the amount of precipitate or undissolved matter.](image)

The froth stability with SDS, DAH and DN2 was considerably higher at pH 6.0 than at pH 9.5 (see Figure 4). This might also be due to the difference in the collector solubility. The froth stability represented by the maximum froth height is directly related to the concentration of collector present in water. It increases with an increase in the concentration of collector (see Figure 4). A similar observation was noted for water recovery. In contrast, changing pH from 9.5 to 6.0 had a rather small impact on the froth stability and water recovery with DPC, which
is consistent with the observation that changing pH alone would not change the solubility of DPC.

**Jameson cell gave better flotation performance than mechanical cell**

A step-wise optimisation can lead to effective flotation separation of difficult-to-harvest microalgae. In a previous study it was demonstrated that a Jameson cell can perform better than a mechanical cell (Garg et al. 2014). On comparing the flotation performance of DPC and DAH using a Jameson cell, Figure 6 shows that DPC not only performed better, with overall recoveries reaching 99%, but it also had a higher enrichment ratio close to 23, compared to 97% recovery with a mere 11 times enrichment ratio for DAH.

![Figure 6. Comparison of flotation performance with different collectors and machines. Shown are algal recovery and enrichment ratio using 15 ppm DPC at pH 9.5 with a Jameson cell or a mechanical cell. The data for 25 ppm DAH at pH 6 were adapted from Garg et al 2014.](image)

This study demonstrates that predominantly choice of collectors based upon the head group present effects the effectiveness of flotation process. This is followed by collector concentration, and the adjustment of pH which helps to increase collector availability and microalgal hydrophobicity. In combination with a suitable flotation machine this can lead to desirable flotation performance of marine microalgae to enable effective dewatering.

**Conclusions**

DPC outperformed SDS, DAH and DN2 in flotation separation of marine microalga *Tetraselmis* sp. M8 from aqueous medium because DPC was able to render the microalgae hydrophobic and produce metastable froth, and had relatively high solubility in
water, indifferent to pH changes. Metastable froth is desirable for microalgae flotation; higher froth stability leads to higher water recovery and thus a lower enrichment ratio. The Jameson flotation outperformed mechanical flotation in separation of marine microalgae from water, and the combined use of DPC and the Jameson cell led to 23-fold increase in algal concentration, with over 99% of marine microalgae recovered.

Acknowledgements

Financial support for this study, provided by the Australian Research Council and a CIEF grant by The University of Queensland, is gratefully acknowledged.

Appendix A. Supplementary data

Dodecyl pyridinium chloride:

N-dodecylpropane-1,3-diamine hydrochloride:

Dodecyl amine hydrochloride:

Sodium dodecyl sulphate:

*Figure A.1. Chemical structures of surfactants tested for froth flotation.*
Figure A.2. The Microalgal recovery (Y) and enrichment ratio (ER) for different collectors at 10 ppm and 25 ppm. Error bars represent the standard error of the mean values from three replicates.

References


6. CHEMICAL-FREE FROTH FLOTATION OF MORE HYDROPHOBIC MICROALGAE

Background

The study carried out during the PhD helped to refine the flotation process and to gain a deeper understanding on how marine microalgae can be harvested by using froth flotation. During the study it was also found that hydrophobicity plays a key role in microalgae recovery. Different microalgae possess different hydrophobicity. Generally, chemicals are required to increase the efficiency of the flotation process. However, the use of chemicals restricts the application of harvested biomass and makes it unfit for feed or food purposes. During the hydrophobicity testing of different microalgae, some were found to possess a naturally higher hydrophobicity. Hence the comparative study was needed to identify the microalgal strains that can be floated without the addition of chemicals.

Key findings

- *D. salina* microalgae cannot bear the shear force exerted by the process of flotation.
- Microalgae with naturally high hydrophobicity can be harvested efficiently without using toxic chemicals.
- Leftover algae cell and water in the flotation cell (tailing) from the flotation process can be re-cultured.
CHEMICAL FREE FROTHER FLOTATION OF HYDROPHOBIC MICROALGAE

Introduction

Microalgae are photosynthetic organisms with great potential to harvest sunlight and convert carbon dioxide into biofuels, health food and animal feed [1, 2]. In the past few years the potential use of microalgae as animal feed has been considered [3]. Using microalgae as an alternative source of protein cattle can be an economical and environmentally friendly solution [4]. Various evaluations demonstrated the suitability of microalgal biomass as a valuable feed supplement to conventional protein sources such as soybean meal, fish meal, rice bran, etc. [3]. When considering microalgae for protein production, there are three species most commonly used: Chlorella, with 55% protein content [4], Scenedesmus with 50-56% protein content and Dunaliella, with 57% protein content [5]. These algae have higher commercial value as they are not only high in protein composition, but also have significant carbohydrate and lipid content. Generally, microalgal cultures meet or exceed the amino acid profile recommended by the WHO [6].

There are large-scale microalgae cultivation farms around the world producing microalgae for biofuel, nutraceuticals and animal feed. However; the number of such farms is limited. Till today, commercial scale microalgae production for animal feed is not economical, thus there is a limited number of commercial algae farms around the world. One of the major impediments causing such restriction is the dilute concentration of microalgae in the culture water which involves microalgae to be dewatered before they can be fed to animals. As a result, the microalgal dewatering process is recognised as a major impediment towards industrial-scale manufacturing of microalgal bio-products [7, 8]. The selection of suitable algae harvesting techniques depends largely on the microalgal species and the desired final product [7, 9]. Flotation technique with its relatively rapid operation, low space requirements, high flexibility and moderate operational cost, has the potential to overcome the bottleneck of feasible microalgal harvesting [10].

Considering the performance of flotation in mineral separation, it is a proven technology to effectively capture small particles from aqueous solutions using gas bubbles [11]. Efficient flotation relies on successful collision and attachment of bubbles and particles [12]. Dispersed air flotation (DiAF), has been widely used to upgrade coal and minerals at large scale (cell volumes reaching up to 500 m$^3$). DiAF seems to be an economical and efficient technique for harvesting of microalgae [13].
The present study demonstrates that flotation can be successfully carried out for marine as well as fresh water microalgae [13, 14]. The study also highlights that the surface hydrophobicity of microalgal cells plays a key role. If microalgae are not hydrophobic, then the hydrophobicity can be rendered by adding chemicals. However, the use of chemicals to modulate hydrophobicity is not acceptable if the end product from microalgae is to be used as a food additive for animal or human consumption.

Different microalgae differ in their natural hydrophobicity. In the present work, five different microalgal strains were screened and their natural hydrophobicity was measured. Some of the screened microalgae had high natural hydrophobicity. Since hydrophobicity is key parameter for successful flotation, a chemical free flotation test for each microalga was carried out. Tailings from the flotation test were also tested for their microalga recultivation efficiency. Results helped to develop chemical free efficient dewatering techniques for microalgae which could result in commercial production of animal feed-grade biomass.

**Materials and Method**

**Algal culture and characterisation**

The marine microalga *Tetraselmis* sp. M8 was isolated from the Sunshine Coast, Queensland, Australia (26°39′39″S, 153°6′18″E; Genbank accession number JQ423158) and the freshwater microalga *Chlorella* sp. BR2 was isolated from the Brisbane River, Tennyson, Queensland, Australia (−27°31′21.36″S, 153°0′32.87″E; Genbank accession number JQ423156; *D. salina* and *Haemotococcus* sp. were collected from MBD, Townsville whereas, *Scenedesmus* sp. NT8c was collected from Gamba dam at Douglas Daily Research Farm, Winellie, Northern Territory, Australia. Marine microalga *Tetraselmis* sp. M8, *D. salina* sp. and *Chlorella* sp. BR2 were cultivated in silicate free f/2 medium at 35 ppt, 60 ppt and 0 ppt salt concentration. Fresh water microalgae were cultivated using Bold’s Basal Medium (BBM), under 120 μmol photon m⁻² s⁻¹ with 16/8 hrs light/dark cycles, at 26 °C ± 1 °C in 2 L-Erlenmeyer flasks with continuous air and nutrient supplies. When the microalgae reached the exponential growth phase, samples were collected for flotation experiments.

**Modified BATH test for hydrophobicity measurement**

The hydrophobicity of microalgae was measured by using the modified adherence-to-hydrocarbon method [15] as mentioned previously [14].
Froth flotation

Flotation experiments were carried in the absence of collector. A procedure was followed as previously reported [14]. The microalga recovery (Y) and water recovery (WR) were determined using the following equation:

\[ Y = 1 - \frac{T}{F} \]  

\[ WR = 1 - \frac{T}{F} \]

Where, \( T \) is the mass of tail left in the flotation cell, \( F \) is the mass of feed, \( t \) is the microalgae concentration in the tail, and \( f \) is the microalgae concentration in the feed.

The enrichment ratio (ER) is defined as the ratio of the concentration of algae in the concentrate to the concentration of algae in the feed. It was calculated using the following formula:

\[ ER = \frac{Y}{WR} \]

Recultivation

The tailings of *D. salina*, *Scenedesmus* sp., *Chlorella* sp. from flotation experiments were re-cultured in 2 L-Erlenmeyer flasks by adding medium and 1 L of water. The cultures were cultivated under 120 \( \mu \)mol photon m\(^{-2} \) s\(^{-1} \) with 12-h light/dark cycles, at 21°C ± 1°C. The cultures were aerated continuously.

Results

Hydrophobicity testing using hexane

On comparing the hydrophobicity of five different microalgae at a natural pH of around 9.3 (±.03), it was observed that each microalga showed different hydrophobicity (figure 1). *Tetraselmis* sp. was the least hydrophobic (1.6%) whereas, *Chlorella* sp. (29%) was the most hydrophobic.
On performing flotation tests on each microalga, a clear trend in the flotation performance was observed (figure 1). As the hydrophobicity of different microalgae increased, the overall microalgae recovery also increased. *Chlorella* sp. had maximum hydrophobicity (figure 1) and performed best with maximum overall recovery of 90% (figure 1) followed by *Scenedesmus* sp. with more than 66% recovery. *Tetraselmis* sp. had the lowest recovery value of 6%. *D. salina* and *Haemotococcus* sp. had 34% and 11% recovery values, respectively. The presence or absence of salt in the water did not have an effect on the overall flotation performance.

Figure 1. Algae biomass recovery for different microalgae using flotation method

The data in figure 2 represents the volume of water recovered during the flotation process for five different microalgae. According to Eq. (3), the higher the water recovery, the lower the enrichment ratio would be. Since *Tetraselmis* sp., *D. salina* and *Haemotococcus* sp. had higher water recoveries and lower algal recoveries, their enrichment ratios were lower, ranging merely between 1 and 4 times (figures 3 and 4). On the other hand, *Chlorella* sp. and *Scenedesmus* sp. performed better in terms of algae recovery and also resulted in a lower water recovery as a result of which they showed higher enrichment ratios, ranging between 16 and 22 times (figure 4).
Chemical free froth flotation of *D. salina*

*Dunaliella salina* may be somewhat unique among the various algae in that it does not have a true cell wall. Instead, *D. salina* may be considered to have a protective phospholipid membrane that is ruptured easily [16]. Considering that the absence of a rigid cell wall makes *D. salina* fragile, it was expected that hexane as a strong solvent it was expected that hexane may rupture the cells during hydrophobicity testing. In order to confirm this hypothesis, chlorophyll concentration in medium before and after the BATH test was measured. Chlorophyll A and B contents of the modified BATH test samples were measured and compared with control samples. It was observed that modified BATH test samples had chlorophyll A and B present in the supernatant. This suggests degradation of cell membranes and rupturing of the cells. The remaining four microalgal strains showed
no cell rupturing when their hydrophobicity was measured using the modified BATH test during the flotation process.

Figure 4. Chlorophyll A and B concentration in control and hydrophobicity test samples.

Re-cultivation of microalgae

Since the concentrate collected from the flotation run displayed cell rupturing due to sheer forces of bursting air bubbles (figure 4); there were concerns for the condition of cells in the tailing. A recultivation from tailing experiment was carried out for D. salina. Figure 5 represents the total biomass of D. salina on day 0 and 7. The alga was cultured using tails from the flotation process. An increase in biomass indicates that there was no damage to the algae that were collected in the tailings or that cells were able to repair themselves. On comparing the recultivation of tailings from Chlorella sp. and Scenedesmus sp. a similar result was obtained (figure 6). Due to poor performance of Tetraselmis sp. and Haemotococcus sp. they were not recultivated.

Figure 5. Overall biomass of D. salina during the start each cultivation cycle from tailings and at the end of the cultivation phase.
Figure 6. Overall biomass of Chlorella sp. (left) and Scenedesmus sp. (right) during the start of each cultivation cycle from tailings and at end of the cultivation phase.

Conclusion

The overall results indicated that a chemical free flotation process is not the optimal harvesting method for Tetraselmis sp. and Haemotococcus sp. As these two algae are less hydrophobic, there was no adequate attachment of microalgae to air bubbles. Thus their recovery was low. Based on the modified BATH tests and the flotation tests, it was observed that the overall hydrophobicity of D. salina was comparatively higher. But this was likely due to the absence of rigid cell walls and the algae were not able to overcome the sheer pressure exerted by the process of flotation, thus resulting in cell mortality. On the other hand, Chlorella sp. and Scenedesmus sp. were better performers. They did not only show higher recovery rates, but also had the lowest water recovery which resulted in the highest enrichment ratio of the microalgae tested in this study.

The results are promising and could help to develop a rapid, yet cost-effective dewatering technique for harvesting microalgal biomass which can be used as a protein feed stock supplement. The techniques developed using flotation would help in continuous harvesting of biomass, while tailings can be used to re-culture algae. The process could be easily scaled up and, if used at mining scale, is capable of processing volumes anywhere between 1000 L to 1,000,000 L per hour.

Reference


7. Conclusion and Future Prospects

Conclusion

Early findings of this project demonstrated the potential for optimization of microalgal strain cultivation and harvesting. They emphasized that a locally isolated strain would likely adapt better to local changing environmental conditions and provide a more stable and productive culture. Initial lab based screening of several microalgal species from the collection helped in selecting the microalgae.

A comparison of indoor laboratory conditions to mid-scale (1000 L) outdoor conditions showed that lipid productivity was more than doubled under outdoor conditions. However, commercially feasible production of microalgal biodiesel would require a biorefinery approach to produce biodiesel as well as other value-added products such omega-3 fatty acids and protein-rich biomass.

Although extensive studies have been carried out on open and closed cultivation methods, very little work has been carried out on two-stage hybrid cultivation systems. The results confirm that two-stage hybrid cultivation systems have the potential to improve microalgal lipid productivity, both under volumetric as well as areal consideration. The average growth rate of the split system was higher than that of both single systems (photobioreactor or open pond). When comparing the doubling time; the split system showed a significantly higher growth rate and shorter doubling time. However, a split cultivation system may result in higher construction and operating costs due to additional requirements for liquid handling.

As a whole, the techniques used for Tetravelmis sp. (M8) isolation, selection and mid-scale cultivation demonstrate that they can lead to the identification of microalgal strains with potential for large-scale cultivation. Additional factors to be considered for large-scale production include harvesting, currently a major cost factor due to the use of centrifuges; one of the possible alternative techniques could be froth flotation.

Similar to mineral flotation, also in this study the most important mechanism for successful flotation was found to be the attachment of the microalgae to air bubbles. The flotation kinetics in absence of collector for Chlorella sp. (BR2) in freshwater medium and Tetravelmis sp. (M8) in seawater medium were quite distinct.
Furthermore, no significant difference in flotation performance in the presence or absence of salts for freshwater microalgae (*Chlorella* sp.) as well as marine microalgae (*Tetraselmis* sp.) demonstrates that ionic strength plays a rather minor role in determining the flotation of microalgae. The flotation performance of the marine microalga was poorer than that of the freshwater microalga because the former had a lower degree of hydrophobicity. This finding signifies that not all colliding particles attach to the bubbles.

Thus, hydrophobicity plays a key role when considering harvesting microalgae using froth flotation. The good correlation between microalgal flotation recovery and hydrophobicity suggests that one can increase the flotation performance of microalgae by using effective collectors which can render microalgal surface more hydrophobic. Quantification of hydrophobicity for microalgae presents a simple and effective assay that allows screening of flotation collectors and other chemical conditions of microalgal cultures, prior to using elaborate froth flotation assays.

Since all hydrophobic particles attach to bubbles, the physical properties of bubbles also play an important role in determining the overall efficiency of flotation. Separation efficiency varies inversely with bubble size; furthermore small bubbles have higher surface area to volume ratios. Hence, they aid in rapid and efficient particle flotation.

The Jameson Cell employs a plunging jet to produce smaller air bubbles than mechanical flotation cells. These smaller bubbles can interact with microalgae at higher collision probability, higher attachment probability and lower detachment probability, lower ascending rate and higher free surface energy, which are all desirable for flotation. However, other properties of the Jameson Cell such as recycling ratio adjustment, froth height and air to pulp ratio may also contribute.

The flotation performance of microalgae can be increased by using more effective collectors that can increase the hydrophobicity of microalgal surfaces. When considering less hydrophobic algae, the collector plays a key role in rendering them more hydrophobic which in turn improves the biomass recovery by froth flotation. Studying the effect of pH on the functioning of collector indicated that solubility of different collectors differ at a given pH value. To get the most efficient recovery at higher pH it is important to select the collector that is able to dissolve completely. The results suggest that control of surfactant solubility is important for cationic flotation of microalgae. The Influence of pH on the collector has been discovered as a missing
link between surfactant precipitation and flotation efficiency was identified. In case of
the marine microalga M8, surfactant precipitation is detrimental to the flotation.

Higher froth stability led to higher water recovery and thus lower enrichment ratio.
Hence control of froth stability in microalgae flotation is important for achieving a high
enrichment ratio. Metastable froth is desirable for microalgae flotation. For a given
surfactant, a low enrichment ratio of microalgae flotation is accompanied by high water
recovery and high froth stability.

When considering algae with sufficiently higher natural hydrophobicity, flotation would be a
more cost-effective method for primary dewatering compared to centrifugation; however,
centrifugation may still be required for secondary dewatering of the concentrated algae
prior to drying.
Future prospects

There are a number of areas where research is still needed. Even though the microalgae cultivation data collected are representative of winter and spring months, this testing should be completed over the course of the year. Summer is when average temperatures would be higher; how well microalgae can grow in higher temperatures should also be observed.

Since the air lift system was used for mixing microalgae in the raceway pond, there is a need to carry out a more comprehensive study wherein paddle wheel and airlift systems are compared side-by-side and their effect on microalgae growth and mixing is assessed.

When considering the use of flotation for harvesting of less hydrophobic microalgae, more tests to the impact of the frothing properties of the surfactants on flotation recovery should be studied. In addition the environmental impacts of chemical dosing rates should be completed on a batch culture of algae grown in a raceway. Tests should also be performed to determine the potential of recycling collectors for example DPC. By doing so, the overall operational cost of the process can be reduced and the overall environmental impact will also be smaller.

Batch tests were carried out using a laboratory-scale flotation cell; however, it is necessary to replicate what was found on a small scale. Thus, the pilot flotation rig should be operated with the optimum conditions as well as the dosing rates that were found with the laboratory rig. Also, since the pilot rig would be used in continuous mode; conditions will differ from laboratory test conditions; hence, the pilot rig should be run with varying DPC doses to find optimum conditions for continuous mode.

Once the operating conditions for the machine are optimised there will be a need to complete research for the proper molar ratio of algae to DPC. The optimum DPC dose found in this project of 15 mg/L depended on the concentration of algae in the culture medium (0.6 to 0.8 g/L). DPC is able to harvest the algae because it is able to physically bind to algae and impart hydrophobicity to each individual algal cell. If a raceway system were used, in order to bring the algae concentration up to 1 to 1.2 g/L, much more DPC would be needed. Research into the optimum molar ratio of algae to DPC would need to be completed before a higher feedstock is run through the Jameson cell unit.
Finally, chemical free flotation carried in the laboratory for production of biomass gave promising results. Preliminary studies are being carried out by the Algae Biotechnology Laboratory in collaboration with Xstrata technologies. The results from this should be analysed and the potential application of the technique at commercial scale should be explored.

Further investigation and carrying out the above mentioned studies will demonstrate the capability of the dispersed air flotation process as a rapid, yet cost-effective process for primary dewatering of microalgae for large-scale biomass production.
APPENDIX

AN OVERVIEW OF ALGAL FUELS (ACCEPTED BOOK CHAPTER)

Sourabh Garg, Rakesh R. Narala and Peer M. Schenk

Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

Sourabh Garg: s.garg@uq.edu.au; Rakesh Reddy Narala: r.narala@uq.edu.au;

* Corresponding author; e-mail address: p.schenk@uq.edu.au

Abstract

In order to address energy security and climate change there has been an increase in the demand for biofuel globally. The production of first generation biofuels using plant-based material directly competes for arable land and biodiverse landscapes, thereby raising concerns for food security and the natural environment. Microalgae are considered a promising candidate for production of biofuels as well for CO₂ remediation. They are capable of producing feedstock for biodiesel, bioethanol, biohydrogen and biogas in nearly any type of water without the need to compete for arable land. Various studies have shown combustion of biofuel to be equivalent or more environmentally friendly when compared to fossil fuels, however their production is not yet economically viable. This chapter provides an overview of different types of biofuels produced by microalgae and points towards future developments.

Introduction

The increased consumption of fossil fuels and greenhouse gas emissions are causing turmoil with deleterious changes in the environment, emphasizing the need for alternative and renewable fuels to become a reality. Global energy demands are currently met either by burning fossil fuel such as coal, oil and gas or by using nuclear power. It is evident that out of all the fossil fuels, liquid fuel derived from crude oil continues to be the primary source of energy throughout the world to run motor vehicles as approximately more than 1.5 trillion barrels of oil has been consumed since 1859 [1-4]. Though the production rate of liquid fossil fuel is not increasing each year, the amount of fuel consumed is increasing, thus widening the gap between the supply and demand [1].
Due to excessive use of fossil fuel, we face three major problems: a) the imminent depletion of natural fossil fuel reserves, b) global climate change due to excessive release of greenhouse gases such as CO$_2$ [5], c) increased competition between economies; further leading to political rivalry, oil wars and destabilized economy [6]. Therefore alternative methods and more sustainable sources of energy production are required. New sources of energy should not only be cost effective but also carbon neutral and renewable. Nearly all renewable sources of energy (e.g. solar, wind, hydro, geothermal, etc) provide electricity, but approx. 80% of current energy supplies are dependent on fossil fuel. Even with the anticipated transition to electric cars, many industries, trucks, ships and planes will still need to be powered by fuel. Hence there is an urgent need for the development of renewable fuels that can be utilized in the transportation sector as well as other uses [7, 8]. In the past few years, immense research efforts had been made on improving the conventional renewable source of energy such as solar, wind, hydro and geothermal power. One option is to store the generated electricity in the form of fuels (e.g. as hydrogen or methane). Considering that the present market is oil-driven and we cannot depend on conventional resources there is a need to develop alternative liquid fuels [9, 10]. Biofuels are chemically modified compounds produced from various biological materials derived from plants, animals or microorganisms that in the future may be able to cover a substantial proportion of the fuel demand [9].

Biofuels

Carbon in primary-sourced biofuels is derived from photosynthesis which involves the conversion of free carbon dioxide from the atmosphere into metabolites and compounds that are stored in various organic forms, such as starch (from corn, wheat, barley, potatoes etc.); sugar (in plants like sugarcane, beets, fruits, etc); cellulose or lignin (from agriculture residues, trees); fats or lipids. Starch and sugar can be converted into bioethanol; cellulose and lignin can be converted into alcohols and methane, whereas fats and lipids can be converted into biodiesel [11, 12].

Biofuels produced from these biological materials or waste oil can be close to carbon-neutral which reduces the amount of greenhouse gases and thereby help in preventing climate change [3]. However, often fossil fuels are involved in the highly-mechanized cultivation of biofuel crops that require fertilizers and pesticides for growth and then machines for harvesting, transport and the extraction of feedstock before finally being converted to biofuels; all of these processes currently depend strongly on fossil fuel.
Biodiesel is mainly produced in Europe and bioethanol is mainly produced in Brazil and USA [13-15].

Table 1 compares the energy content and some physical properties of fossil fuels and biofuels. On comparing one liter of petrol to similar volumes of different biofuels, the energy content of most of the biofuels is equivalent or higher than that of conventional petrol and produces less carbon dioxide upon combustion.

Table 1. Comparison between fossil fuels and plant-derived biofuels.

<table>
<thead>
<tr>
<th>Fuel type</th>
<th>Chemical formula</th>
<th>Energy equivalent of petrol</th>
<th>Energy content</th>
<th>Flash point °C</th>
<th>CO2 Emissions (per kg of fuel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petrol</td>
<td>C4 to C12</td>
<td>100 %</td>
<td>4.4 to 4.9 MJ/Kg</td>
<td>-42.77</td>
<td>3.09 kg</td>
</tr>
<tr>
<td>Diesel</td>
<td>C8 to C25</td>
<td>113 %</td>
<td>4.3 to 4.6 MJ/Kg</td>
<td>73.88</td>
<td>3.17 kg</td>
</tr>
<tr>
<td>Biodiesel</td>
<td>Methyl esters of C12 to C22 fatty acids</td>
<td>103 %</td>
<td>3.8 to 4.1 MJ/Kg</td>
<td>100 - 170</td>
<td>2.5 kg</td>
</tr>
<tr>
<td>Ethanol</td>
<td>CH3CH2OH</td>
<td>96.7% (E10)</td>
<td>2.7 to 3.0 MJ/Kg</td>
<td>12.77</td>
<td>3.06 kg (E 10)</td>
</tr>
<tr>
<td>Methane (compressed)</td>
<td>CH4</td>
<td>133%</td>
<td>4.7 to 5.2 MJ/Kg</td>
<td>-184.44</td>
<td>-</td>
</tr>
<tr>
<td>Hydrogen (compressed)</td>
<td>H2</td>
<td>269 %</td>
<td>12.0 to 14.2 MJ/Kg</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Biofuels are primarily produced from plants such as oil palm, rapeseed, soybeans, sugarcane, and corn [9, 16]. Biofuel produced from these crops are known as first generation biofuels [8], however due to the ever increasing demand for food and a shortage of arable land, it is highly questionable to use edible crops for the production of biofuels [17]. The immediate solution to these problems is the use of non-edible crops and microalgae, ideally that do not compete with arable land. Biofuel produced from Jatropha seeds, used cooking oil, soap stock, grease and tallow are known as second generation biofuels [13, 16, 18] but, even these sources are not enough to meet the world’s oil demand. When compared to all of the present sources, microalgae are often considered the most promising candidates to produce biofuels [8, 9, 16, 19] Microalgae, even at relatively low production rates, are at least twice more productive than any other biofuel crop, while potentially not competing with arable land.

**Microalgae**

Microalgae are microscopic mostly single-cellular organisms that have been cultivated commercially for food, animal feed, cosmetics and API (Active Pharmaceutical Ingredients)
for many decades [19]. Unicellular microalgae have a lower footprint as compared to higher plants [20]. Moreover, it is often mentioned that they do not compete with edible crops for water and land as they can be grown on desert terrain using sea, brackish or waste water [8, 9, 16]. Also the photosynthesis process of microalgae is more efficient as compared to higher plants. The doubling time of rapidly-growing microalgae is approximately 24 hours, once they reach exponential phase. Some of the microalgae can even double in numbers in as little as 3.5 hours [16]. Microalgae can be cultivated in closed systems such as photo-bioreactors or in open ponds raceways.

Microalgal biomass contains metabolites like carbohydrates, proteins, lipids which can be used to produce various types of biofuels such as biohydrogen, bioethanol, biodiesel; healthcare products and animal feed [20-22]. The oil content of microalgae can range between 15% and 40% of dry weight (DW), but some microalgae may contain up to 80% oil or hydrocarbons which can be extracted through a single method or by a combination of methods [16]. Cell debris (by-products) left after producing biodiesel can be used for producing biogas by anaerobic fermentation. This biogas can also be used to produce electricity [23], see Figure 1. Thus ideally, biofuels derived from microalgae could reduce the carbon emission, because energy consumed for producing biodiesel can either come from biodiesel or the biogas produced from biomass residue [23]. Water and CO$_2$ can be recycled and used for further cultivation. This CO$_2$ can be atmospheric CO$_2$, but actual bioremediation (biological carbon capture and sequestration) can only be achieved if biomass is used for the production of stable carbon, such as biochar that can be stored underground, so that a net reduction of CO$_2$ can occur. Interestingly, biofuel production from microalgae can also be nearly independent from fertilizer input, when nutrient-rich leachate from anaerobic digestion is re-used to grow more algae (Fig. 1).
Biodiesel

Research reveals that higher operating pressure and combustion temperature of diesel engines make them 30 to 40% more efficient when compared to petrol engines [9]. Moreover, studies by Khan et al. (2009) indicate that triacylglycerides (TAGs)-derived biodiesel is a promising alternative fuel and is more environmentally-friendly as its production from biomass can be near CO₂ neutral, making biodiesel one of the most important biofuels (Table 2) [3].

Table 2. Emissions comparison between biodiesel and petroleum diesel

<table>
<thead>
<tr>
<th>Emissions</th>
<th>Biodiesel</th>
<th>Mixed biodiesel (20% biodiesel and 80% petroleum diesel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regulated emissions (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total unburned hydrocarbons</td>
<td>-93</td>
<td>-30</td>
</tr>
<tr>
<td>Carbon monoxide</td>
<td>-50</td>
<td>-20</td>
</tr>
<tr>
<td>Particulate matter</td>
<td>-30</td>
<td>-22</td>
</tr>
<tr>
<td>NOx</td>
<td>+13</td>
<td>+ 2</td>
</tr>
<tr>
<td>Non-regulated emissions (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphates</td>
<td>-100</td>
<td>-20</td>
</tr>
<tr>
<td>Polycyclic aromatic hydrocarbons (PAHs)</td>
<td>-80</td>
<td>-13</td>
</tr>
<tr>
<td>Nitrate PAHs (NPAHs)</td>
<td>-90</td>
<td>-50</td>
</tr>
<tr>
<td>Ozone depletion potential (ODP) of Hydrocarbon</td>
<td>-50</td>
<td>-10</td>
</tr>
<tr>
<td>Life cycle emissions (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>-80</td>
<td></td>
</tr>
<tr>
<td>Sulphur dioxide</td>
<td>-100</td>
<td></td>
</tr>
</tbody>
</table>

(-): Less % of pollutant emission from biodiesel in comparison to 100% petroleum diesel.
More % of pollutant emission from biodiesel in comparison to 100% petroleum diesel, i.e. only in the case of oxides of nitrogen (NOx).

ODP of a given substance is defined as the ratio of global loss of ozone due to given substance over the global loss of ozone due to CFC-11 of the same mass. Here it means that biodiesel has 50% less potential to degrade the ozone layer when compared to petroleum diesel.

PAHs are organic compounds containing only carbon and hydrogen composed of multiple aromatic rings. Adapted from Khan (2009) [3].

Also when compared to ethanol, biodiesel has more advantages such as, a) ethanol is a corrosive liquid which makes it difficult to store and transport, b) while producing ethanol, if the concentration of ethanol exceeds a certain threshold limit, it acts as a process inhibiting factor, c) combustion of biodiesel emits fewer toxic gases when compared to that of bioethanol [3, 24].

Like photosynthetic plants, microalgae store energy mainly in the form of starch and lipids. Lipids are mainly produced when microalgae are subjected to stress, in particular in marine algae. This stress can be of different types, such as nutrient starvation, pH change, temperature shock, UV light, etc. Out of all methods, the most commonly used method is nutrient starvation. [25].

The oil used for making biodiesel primarily consists of TAGs. In the process of transesterification, three molecules of fatty acids are transesterified with three molecules of alcohol (generally methanol) to produce three molecules of methyl esters of fatty acids (FAME) commonly known as biodiesel and a molecule of glycerol. It is a multistep conversion process that needs acids or alkalis or enzymes [26, 27] as catalysts. The concentration of catalyst is about 1% by weight oil [26].

The overall oil content in dry microalgae is between 15 to 50% and can be as high as 80% of its DW. Table 3 shows oil contents among some of the examined algal species.

Table 3. Oil content of various microalgae

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Oil content (% DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botryococcus braunii</td>
<td>25-75</td>
</tr>
<tr>
<td>Chlorella sp.</td>
<td>28-32</td>
</tr>
<tr>
<td>Cryptothecodinium cohnii</td>
<td>20</td>
</tr>
<tr>
<td>Dunaliella primolecta</td>
<td>23</td>
</tr>
<tr>
<td>Isochrysis sp.</td>
<td>25–33</td>
</tr>
<tr>
<td>Monallanthus salina</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Nannochloris sp.</td>
<td>20–35</td>
</tr>
<tr>
<td>Nannochloropsis sp.</td>
<td>31–68</td>
</tr>
</tbody>
</table>
Assuming complete combustion of a biodiesel with C19 molecules, about 2.52 kg of carbon dioxide would be produced for every kilogram of fuel burnt. This is lower than the 3.17 kg of carbon dioxide produced per kilogram of petroleum diesel burnt. However, the energy content for biodiesel is about 38 MJ/kg and it is about 43 MJ/kg for petrodiesel. Thus, we need about 1.13 times more biodiesel than petrodiesel to obtain a given amount of energy. Correcting for this, biodiesel would produce around 2.86 kg of carbon dioxide per kilogram of fuel burnt. This is still better than petrodiesel. The concentration of nitric oxide produced during the combustion of biodiesel can be reduced by varying the temperature at which engines operate, thus reducing the overall emissions [29].

Bioethanol

Ethanol was used as a fuel in conjunction with an oxidizer such as liquid oxygen in early bipropellant rocket vehicles [30]. Bioethanol, produced by sugar fermentation is the most common biofuel used in the USA and Brazil, while biodiesel, trans-esterified mono-alkyl esters of vegetable oil or animal fats, is the most common biofuel used in Europe [13, 15]. Although production of ethanol using crops as feedstock can address environmental problems, it still impacts on food supply and security. The use of food crops for ethanol production would result in an increase in food prices. Several studies report the production of bioethanol from lignocellulosic waste materials, such as farm and cattle waste, leaf and yard waste, as well as industrial and municipal waste [31]. However the use of these raw materials is limited due to their sparse availability, low yield and the high cost of the hydrolysis process. On the other hand, microalgae are considered by some as the ideal candidate for the production of bioethanol. The reason for higher ethanol production in microalgae is higher photon conversion efficiency and the ability to synthesize and accumulate large amounts of carbohydrates [31].

Certain species of microalgae can produce ethanol from photosynthesis when subjected to dark anaerobic fermentation resulting in the direct production of ethanol [31]. Moreover, residual biomass left over after lipid extraction from oleaginous microalgae is rich in starch. These starches can be converted into sugar which can be further converted into ethanol by fermentation [32]. To further make ethanol production from microalgae more
economical, attempts are being made to incorporate ethanol producing genes into microalgal genomes and thereby creating recombinant microalgae [32]. Algenol Biofuels Inc. report that their system can produce 9,000 gallons of ethanol per acre per year (13,6 kL/ha), which is far greater than the yield for ethanol from corn [33]. Table 4 lists some of the microalgae and their starch content.

Table 4. Starch content of various microalgae.

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Starch (%DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlorella vulgaris</em></td>
<td>37.0</td>
</tr>
<tr>
<td><em>Chlorococcum</em> sp. TISTR8583</td>
<td>26.0</td>
</tr>
<tr>
<td><em>Oscillatoria</em> sp. TISTR 8869</td>
<td>19.3</td>
</tr>
<tr>
<td><em>Phormidium angustissimum</em> TISTR 8979</td>
<td>28.5</td>
</tr>
<tr>
<td><em>Scenedesmus acuminatus</em> TISTR 8457</td>
<td>7.3</td>
</tr>
<tr>
<td><em>Spirulina fusiformis</em></td>
<td>37.3–56.1</td>
</tr>
<tr>
<td><em>Scenedesmus</em> sp. TISTR 8579</td>
<td>20.4</td>
</tr>
</tbody>
</table>

Adapted from Rodjaroen (2007) and Rafiqul (2003) [34, 35].

The energy content for ethanol is about 31.1 MJ/kg with an octane number of 129, and for petrol it is about 44.4 MJ/kg with an octane number of at least 91. Whereas for E10 (90% gasoline and 10% ethanol) the energy content is 43.54 MJ/kg with an octane number of 93/94 [30]. Thus, we need about 1.03 times of E10 fuel to get the same amount of energy as petrol.

**Biohydrogen**

Hydrogen as fuel source has tremendous potential and can become a major source of clean and renewable energy. Biohydrogen production from microalgae on commercial scale can be useful as it satisfies most of the criteria required by a clean and renewable energy source [8]. Biohydrogen production from microalgae has been known for more than 65 years. Cyanobacteria and microalgae can produce hydrogen by the process of photo-evolution which is catalyzed by hydrogenases. There are two different processes of hydrogen production from algae.

1. Direct bio-photolysis is a one-step process for a sustained hydrogen evolution under light irradiation. The light energy is absorbed by the pigments at photosystems PSII and PSI, and electrons are transferred from PSII via PSI to ferredoxin. Two molecules of water are broken down to produce oxygen and hydrogen gas [36]:

   \[ 2 \text{H}_2\text{O} + \text{light} = 2 \text{H}_2 + \text{O}_2 \]
2. Indirect bio-photolysis consists of two stages: photosynthesis for carbohydrate accumulation, followed by dark fermentation of the carbohydrate reserve for hydrogen production. By splitting the process in two, production of oxygen and hydrogen gas can be separated. This separation avoids enzyme deactivation and makes hydrogen gas purification easier [36]. The steps involved are as follows:

   Step 1 (growth phase): $\text{6 H}_2\text{O+6 CO}_2+\text{light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6+\text{6 O}_2$

   Step 2 (fermentation phase): $\text{C}_6\text{H}_{12}\text{O}_6+2 \text{ H}_2\text{O} \rightarrow 4 \text{ H}_2+2 \text{ CH}_3\text{COOH}+2 \text{ CO}_2$

   According to Melis and Happe [37], using the two-stage photosynthesis process, a theoretical maximum yield of hydrogen from green algae is about $198 \text{ kg H}_2 \text{ ha}^{-1} \text{ day}^{-1}$ [37]. Table 5 lists some of the known $\text{H}_2$-producing microalgae and their hydrogen yield.

**Biogas**

Algal biomass contains high amounts of carbon, nitrogen and phosphorus. It also contains trace amounts of minerals such as iron, zinc and cobalt [41]. Nitrogen consumption of microalgae could vary from 8 to 16 tons $\text{N ha}^{-1} \text{ year}^{-1}$ [42]. Since the biomass is rich in nutrients, loss of these nutrients may not be economically and environmentally sustainable [43]. Anaerobic digestion of algal biomass can recycle the macro- and micro-nutrients present in algae in the form of fertilizer which his can be used for plants (Fig. 1). The anaerobic digestion not only recycles nutrients but also helps in generation of methane gas which can be used as fuel source or for the generation of heat or electricity.

The entire digestion process occurs in three steps. In the first step, the complex compounds are broken down into soluble sugars by hydrolysis. Then, fermentative bacteria convert these sugars into alcohols, acetic acid, volatile fatty acids, and a gas containing $\text{H}_2$ and $\text{CO}_2$, which is metabolized into primarily $\text{CH}_4$ (60–70%) and $\text{CO}_2$ (40–30%) by methanogens in the last step [44]. The production of biogas using anaerobic digestion is affected by temperature, $\text{pH}$, organic loading and retention time in the reactor. Anaerobic degradability of microalgae can be slow and incomplete and as a result a lower amount of methane is produced compared to digestion of activated sludge [45]. A reason for this may be the robust cell wall of some algae preventing access of the anaerobic bacteria to the biodegradable intracellular material. Table 5 lists some of the microalgae and their methane yield after anaerobic digestion.
Table 5. Hydrogen and methane production capacity of various microalgae

<table>
<thead>
<tr>
<th>Microalgae</th>
<th>Total yield of H₂ per L of algae culture (mL/L/hr)</th>
<th>Methane yield (m³ kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td><em>Chlorella autotrophica</em></td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td><em>Chlorella protothecoides</em></td>
<td>2.93</td>
<td></td>
</tr>
<tr>
<td><em>Chlorella</em> sp. (IOAC085F)</td>
<td>1.33</td>
<td></td>
</tr>
<tr>
<td><em>Nannochloropsis</em> sp.</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td><em>Tetraselmis helgolandica</em></td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td><em>Tetraselmis striata</em> Butcher</td>
<td>0.34</td>
<td></td>
</tr>
<tr>
<td><em>Gracilaria</em> sp.</td>
<td></td>
<td>0.28–0.4</td>
</tr>
<tr>
<td><em>Laminaria</em> sp.</td>
<td></td>
<td>0.26–0.28</td>
</tr>
<tr>
<td><em>Laminaria digitata</em></td>
<td></td>
<td>0.5</td>
</tr>
<tr>
<td><em>Macrocystis</em></td>
<td></td>
<td>0.39–0.41</td>
</tr>
</tbody>
</table>

Adapted from Timmins *et al.* (2009), He *et al.* (2012) and Singh (2010) [38-40].

**Feasibility**

The concept of using microalgae as feedstock for the production of energy dates back to the late 1950’s, but intense research efforts began with the oil crisis in the 1970s [30]. During the last three decades there has been extensive research carried out on algae for biofuel production and CO₂ bioremediation [8].

Based on various studies carried out since the US Department of Energy’s Aquatic Species Program, it is evident that microalgae have a potential to produce a wide range of biofuel and valuable by-products, due to their high productivity of proteins, lipids, carbohydrates, vitamins, pigments and enzymes. There are only a handful of commercial microalgae farms around the world. Major hurdles of microalgae farming include high costs for cultivation, dewatering and labour. There is a need to carry out extensive studies on upstream and downstream processes in order to enable algal fuels compete with existing fuel products [46].

Microalgal derived biofuel have potential in terms of delivering clean and sustainably produced energy for the future without conflicting with food supply as well as damaging forest by deforestation concerns related with first generation biofuels and lignocellulosic processes when using wood as feedstock. Significant cost savings can be expected in the future for (1) improved microalgal strains, (2) improved cultivation systems (better hydrodynamics, mixing/aeration and light distribution), (3) reduced costs for harvesting (e.g. natural settling by gravity, rather than centrifugation), (4) wet oil extraction and recovery technology, and (5) reduced capital and labour costs by scaling up production.
The concept of algal biorefineries, where valuable co-products (e.g. protein-rich animal feed, omega-3 fatty acids, carotenoids) drive economic feasibility, can be envisaged, at least for some time, until algal biofuel production by itself becomes financially viable.

**Conclusion**

Microalgal biofuels not only present a potentially sustainable energy resource and range of by-products but can also help to mitigate CO₂. To meet the current biofuel demand, a large quantity of algal biomass is needed for the production of biodiesel from lipids or of bioethanol from starch. By using a chemical process, algal lipids can be transesterified to biodiesel whose main characteristics are quite similar to those of conventional petroleum diesel, and it can also be blended in any proportion with petroleum diesel. In addition, microalgae have the ability to produce combustible biogases such as H₂, CH₄. These gases can be used in engines and turbines and as feedstock for refineries. Considering the present scenario of biofuel research, it is still too early to choose any single method for biofuel production. However, significant improvements must be made to produce commercially viable biofuel. For this purpose major scientific breakthroughs and investments into large-scale production systems will be required to develop the cost-efficient infrastructure required to scale up algal biofuel production. New techniques need to be developed for rapid harvesting, lipid induction and extraction, as well as for making optimum use of residual biomass.

**Summary**

- The increased consumption of fossil fuels and greenhouse gas emissions are causing turmoil with deleterious changes in the environment.
- Carbon in primary-sourced biofuels is derived from photosynthesis which involves the conversion of free carbon dioxide from the atmosphere into metabolites and compounds that are stored in various organic forms.
- Microalgal biomass contains metabolites like carbohydrates, proteins, lipids which can be used to produce various types of biofuels such as biohydrogen, bioethanol, biodiesel; healthcare products and animal feed.
- Lipids are mainly produced when microalgae are subjected to stress.
Based on various studies carried out since the US Department of Energy’s Aquatic Species Program, it is evident that microalgae have the potential to produce a wide range of biofuels and other valuable by-products.

References


A comparative study: the impact of different lipid extraction methods on current microalgal lipid research

Yan Li1,2*, Forough Ghasemi Naghdi1, Sourabh Garg1, Tania Catalina Adarme-Vega1, Kristofer J Thurecht3, Wael Abdul Ghafor3, Simon Tannock1 and Peer M Schenk1

Abstract
Microalgae cells have the potential to rapidly accumulate lipids, such as triacylglycerides that contain fatty acids important for high value fatty acids (e.g., EPA and DHA) and/or biodiesel production. However, lipid extraction methods for microalgae cells are not well established, and there is currently no standard extraction method for the determination of the fatty acid content of microalgae. This has caused a few problems in microalgal biofuel research due to the bias derived from different extraction methods. Therefore, this study used several extraction methods for fatty acid analysis on marine microalga Tetraselmis sp. M8, aiming to assess the potential impact of different extractions on current microalgal lipid research. These methods included classical Bligh & Dyer lipid extraction, two other chemical extractions using different solvents and sonication, direct saponification and supercritical CO2 extraction. Soxhlet-based extraction was used to weigh out the importance of solvent polarity in the algal oil extraction. Coupled with GC/MS, a Thermogravimetric Analyser was used to improve the quantification of microalgal lipid extractions. Among these extractions, significant differences were observed in both, extract yield and fatty acid composition. The supercritical extraction technique stood out most for effective extraction of microalgal lipids, especially for long chain unsaturated fatty acids. The results highlight the necessity for comparative analyses of microalgae fatty acids and careful choice and validation of analytical methodology in microalgal lipid research.

Keywords: Microalgal oil, Fatty acid, Extract yield, Solvent polarity, Supercritical CO2, Lipid profile

Background
Since the concept of using algae to make fuels was firstly discussed in the 1940s [1], a major focus for research, development and commercialization has become the cultivation of algae for the production of oil (lipid)-based products, in particular biodiesel through lipid transesterification. Algal lipids can be divided into two major types: polar lipids such as phospholipids and glycolipids, and neutral/non-polar lipids such as mono-, di- and tri-acylglycerides and carotenoids based on their physiochemical characteristics [2,3]. Some of these substances have been intensively studied, not only as biofuel feedstock, but also as beneficial food additives and other high-value products (e.g., eicosapentaenoic acid (C20:5 n-3, EPA), docosahexaenoic acid (C22:6 n-3, DHA) and other long-chain polyunsaturated fatty acids (LC-PUFA)) [4,5]. Therefore, there is mounting interest on investigation of microalgal potential for production of food commodities and fatty acids bound as triglycerides for nutraceutical efficacy in recent decades [6]. Significant advances have been made in upstream processing to generate cellular biomass for lipid yields. However, as part of the downstream process, lipid extraction continues to be a significant challenge towards the commercial production of microalgal oil production, even though a multitude of extraction methods have been described in the literatures.

For microalgal oil extraction, although an appropriate technique of cell disruption is a prerequisite [7,8], the efficient extraction of lipids is highly dependent on the
polarity of the organic solvent or solvent mixture used [9,10]. In general, solvent mixtures containing a polar and a non-polar solvent could extract a greater amount of lipids [11]. For example, a combination of chloroform (non-polar), methanol (polar) and water, known as the Bligh & Dyer method, has been used for lipid extraction from a wide range of biological samples [11]. However, concerns about biosafety issues using extraction solvents has driven a demand for biocompatible and less or non-toxic solvents (e.g., dichloromethane) [12]. Alternative solvent methods for lipid extraction thereby have been studied; for instance, saponification has resulted in significant lipid recoveries from several types of microalgae [7,13-16]. In recent years, supercritical fluid technology has been adopted for microalgae oil extraction, especially for pharmaceutical and nutraceutical bioproducts. In comparison with liquid solvent extractions, the supercritical fluid carbon dioxide (ScCO₂) technique offers several advantages, such as no toxicity, no oxidation or thermal degradation of extracts, high diffusivity and easy separation of desired bioproducts [16-18]. However, it has been reported that lipid yield using ScCO₂ extraction was much lower than employing the Bligh & Dyer method on heterotrophically cultured microalgae of Cryptothecodinium cohnii [19]. At present, comparative economics of technical and physiochemical methods for oil extraction have not been accomplished on microalgae cells. Given the large diversity of microalgae species, the ability to successfully and effectively extract oil from cellular biomass becomes paramount in determining the yield and suitability across oleaginous strains [18,20]. However, the current research attention towards oil extraction from microalgae has been predominantly focused on the potential energy efficiency and cost effectiveness of the methods themselves. Despite the differences in extraction efficiency obtained depending on different extraction methods [10-12,21,22], there is little attention on the bias potentially derived from different extraction methods, in particular when screening optimal microalgal species for lipid-based bioproducts. Due to the lack of a standard extraction method for fatty acids (FA) analysis, therefore, the motivation behind this study was to investigate the potential impact of different lipid extraction methods on microalgal lipid research. The present work includes a comparative study of lipid extractions from lyophilised biomass of the oleaginous green alga Tetraselmis sp. Soxhlet extraction was conducted for lipid recovery using either single solvents or mixtures. In addition, algal biomass was used for five different extraction methods that were successfully used for efficient algal lipid extraction in previous studies. These parallel extraction methods were: (1) the monophasic ternary system of chloroform:methanol:water, one of the most commonly used methods for lipid extractions [23]; (2) a less hazardous solvent mixture of dichloromethane:methanol [12]; (3) another alternative solvent mixture of propan-2-ol:cyclohexanewater recommended by Schlechtriem et al. [24]; (4) direct saponification using KOH in ethanol [7] and (5) supercritical CO₂ extraction [25]. We discuss and draw some parallels with these extractions to highlight the differences on extractable lipid production and hydrolysed fatty acid methyl ester profiles on microalgal cells.

Results and discussion
The impact of solvent polarity on lipid extraction
The results obtained for Soxhlet extraction of microalgal lipids showed a significant difference in extraction efficiency between hexane and the mixture of hexane and ethanol in both, total lipids and total FAMES, as well as each individual fatty acid (P < 0.05, Figure 1A and B). As ethanol is a polar solvent, it can extract more polar lipids and likely penetrate the cell wall, hence making triacylglycerides (TAGs; neutral lipids) more available for the non-polar solvent hexane. The lipid extraction yield in the mixture was nearly three times higher than when using hexane alone (Figure 1A). Coincident with the reports of Ryckeboch et al. [11] and Lewis et al. [10], it seems that extraction solvents containing a mixture of a polar and a nonpolar solvent could extract higher amounts of lipids and also some other compounds (e.g., pigments, carbohydrates and algaenans) [26]. Interestingly, this conclusion contradicts the study of Shen et al. [27] stating that 1:1 (v/v) of hexane and ethanol had less lipid yields than hexane on Scenedesmus dimorphus and Chlorella protothecoides. Regardless of the biological difference of these algal species and its resulting different lipid class compositions, the contradiction is possibly also related to the different proportions of hexane:ethanol in the mixture (3:1 vs. 1:1). A similar result was obtained using other mixtures as well, such as chloroform:methanol [11] and hexane-hydroalcoholic solution [28] where different ratios of solvents also resulted in the different extraction efficiencies on microagal lipid extraction. Therefore, it is implied that only appropriate proportions of polar and nonpolar solvents could achieve higher yields of lipid compared with single solvent extraction. Although the Soxhlet extraction method has been used for a range of biological organisms [18,29,30], Soxhlet extraction is extremely time-consuming [22,30]. It also could cause thermo-degradation of LC-PUFAs (e.g., ω-3 fatty acids) [29]. Although the efficiency of Soxhlet extraction could be improved significantly by using solvent mixtures, the extraction yields (percentage of extracts in algal dry weight) were still lower than the values obtained in the parallel extractions (Table 1). The inefficiency of Soxhlet extraction has also been reported in other studies [e.g.,3,22]. Therefore, the Soxhlet extraction method was excluded in the lipid extraction comparison in this study.
Determination of lipid content in the microalgal dry biomass

The Thermogravity Analyser (TGA) measures the change of weight of various materials at given temperatures while the temperature is increased over time [31]. Through the comparison between initial and defatted biomasses, the temperature range of TGA selected in this study was correlated to the lipid content in microalgae. Meanwhile, it is worth noting that there was still a bit of moisture content in the lyophilised biomass within 25-190°C, showing the difference before and after lipid extraction. Therefore, normalised microalgal biomass (via water deduction) is more appropriate for lipid quantification, which is different from conventional gravity measurements. As the accuracy in TGA analysis of algal biomass can reach microgram levels, the application of TGA will be a useful analytical framework for assessing lipid yields from microalgae, especially for microalgal biodiesel research [26].

Our data show that the production of lipid extracts was significantly different among the five extraction methods tested ($P = 0.029$, Table 1). The mean value of lipid content was between 9.4% and 15.05% in lyophilised Tetraselmis sp. M8 biomass. The yield obtained from the mixture of dichloromethane and methanol (Dic:Met), was much higher than those from direct saponification (Eth:KOH) and supercritical-CO$_2$ extractions (ScCO$_2$, $P < 0.05$). The extraction yield from the propan-2-ol and cyclohexane method (Pro:Hex) was also significantly higher than that from Eth:KOH ($P < 0.05$). The extraction yield from the

**Figure 1** Comparison of lipid recovery by Soxhlet extraction utilising hexane and hexane-ethanol (3:1) for A) Lipid yields and B) FAMEs profile. Different letters represent a significant difference between hexane and hexane-ethanol ($P < 0.05$).

**Table 1** Comparison of extract content between different lipid extraction methods

<table>
<thead>
<tr>
<th></th>
<th>Chl:Met</th>
<th>Dic:Met</th>
<th>Pro:Hex</th>
<th>Eth:KOH</th>
<th>ScCO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract content (% of dry weight)</td>
<td>11.66 ± 1.16 (abc)</td>
<td>15.05 ± 0.46 (a)</td>
<td>13.35 ± 1.15 (ab)</td>
<td>9.40 ± 1.64 (c)</td>
<td>10.88 ± 0.46 (bc)</td>
</tr>
</tbody>
</table>

Different small letters indicate significant differences using one-way ANOVA analysis ($P < 0.05$).

Chl:Met: chloroform and methanol method;
Dic:Met: dichloromethane and methanol method;
Pro:Hex: propan-2-ol and cyclohexane method;
Eth:KOH: ethanol and KOH method;
ScCO$_2$: supercritical-CO$_2$ extraction method.
Bligh & Dyer method (Chl:Met) was not statistically different to yields from any of the other methods used ($P > 0.05$). In terms of lipid yields, the order of extraction efficiency on *Tetraselmis* sp. M8 could be ranked as Dic:Met > Pro:Hex > Chl:Met > ScCO$_2$ > Eth:KOH. However, this sequence was not applicable for other microalgae. For example, a contradicting result was observed on the microalga *Cryptothecodinium cohnii* that the lipid yield attained from Chl:Met was nearly double that of ScCO$_2$ [19]. Although it is likely associated with a different extraction process, the effectiveness of a lipid extraction method may also be dependent on the microalgal species used [8,10,22]. Differences can be explained by differences in size and in particular cell wall composition. Therefore, a comparative analysis of microalgal fatty acids and choice and validation of analytical methodology are essential for microalgal lipid research.

Interestingly, the sequence of gravimetrically-measured lipid yields was not equivalent to the order of the FA content when quantifying FAME by GC/MS (Table 2). The total fatty acid content determined by GC/MS varied between 6 to 10% of dry weight (DW). In our comparison, the maximum yield of total FA was achieved through ScCO$_2$ (10%), followed by Dic:Met (8.64%), Chl:Met (8.33%), Pro:Hex (8.18%) and Eth:KOH (6.06%). Discrepancies between both methods ranged from 0.88% for ScCO$_2$ to 6.41% for Dic:Met (Table 1, 2). Similar to this study, such a difference was also observed in the oil extract on *Botryococcus braunii* [32], because of the co-extraction of other compounds (e.g., non-polysaccharide biopolymers, polyaldehydes and polyacetals [33]). Although a further investigation will be needed to identify and quantify these components in *Tetraselmis* sp. M8 biomass, it is also in some ways surprising given the many years dedicated by others to elucidating both lipids and the other chemical compounds as “oil/lipid” content in microalgae [26]. Clearly, the amount of co-extracted non-TAGs in the “lipid” fraction varies for different extraction methods and algal strains, making a comparison of lipid yields across different laboratories and microalgal species extremely difficult. Therefore, only the content of FAME identified by GC/MS, was considered as a useful measure to assess lipid production in this study.

The impact of different extraction methods on microalgal fatty acids yield

The saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and PUFA were all obtained in five extraction methods from *Tetraselmis* sp. M8 biomass, but with different yields ($P < 0.05$, Figure 2). A significant difference was only observed between ScCO$_2$ and Eth:KOH, where the FA yields were much lower in the latter ($P < 0.05$, Figure 2). Given the lower FA yields, Eth:KOH clearly shows a lack of competence for FA extraction. However, this could be specific to *Tetraselmis* sp. M8, since direct saponification was quite successful for the lipid yield and better than liquid solvent extractions for other microalgae species, such as *Thraustochytrium* sp. [7], *bocchrys* galbana [21] and *Phaeodactylum tricornutum* [14]. On the other hand, it demonstrates the importance of testing different extraction methods for different microalgae.

Although the results showed no statistically significant difference between Dic:Met, Pro:Hex and ScCO$_2$ ($P > 0.05$), the mean values of FA yields were relatively higher for ScCO$_2$. Additionally, the yield from ScCO$_2$ can be significantly increased by using wet algal paste rather than dry biomass [34]. This is because supercritical CO$_2$ is a non-polar solvent and the water will act as a natural polar co-solvent [22]. As the biomass used in this study was lyophilised, further improvements for ScCO$_2$-based lipid extraction may be achieved through the presence of water that can facilitate polar extractions. It is conceived that ScCO$_2$ is more efficient to extract more FA yield than other methods. More importantly, energy consumed in the drying process can be reduced by using supercritical extraction technology [22], which would be important from a commercial perspective.

Generally yields between Chl:Met, Dic:Met, Pro:Hex and Eth:KOH (excl. the lower amount of SFA in Eth:KOH, Figure 2) did not differ widely. With concerns about the safety and hassles of using chloroform for microalgal biofuel research, this study suggests that dichloromethane could readily replace chloroform for microalgal lipid extraction. This conclusion is also applicable to plant and animal materials for which Dic:Met and the Bligh & Dyer method (Chl:Met) also gained similar FA yields [12]. For *Tetraselmis* sp. M8 lipid extraction, the non-chlorinated solvents, propan-2-ol and cyclohexane present another alternative to the Chl:Met method. This result is consistent with Chl:Met and Pro:Hex lipid extraction data on *Ditylum brightwellii* [24].

The difference of fatty acid methyl esters (FAME) among five extraction methods

Overall, our comparison highlights that different extraction methods not only could lead to different FA yields (Figure 2), but also affect the FA profile to a large extent (Table 2). Only a few fatty acids were not significantly influenced ($P > 0.05$). However, it is worth noting that they were the most abundant FAs in the lipid extracts (approx. 70% of total FAs), such as C16 hexadecanoic (or palmitic) acid, C18:1 (n-9) oleic acid and C18:2 (n-6) octadecadienoic (or linoleic) acid. These FAs are normally treated as the major components for microalgal biodiesel production [34]. Despite of differences in total lipid yield, it is conceived that these different extraction methods maybe less relevant for microalgal biodiesel research, demonstrated by the similar amount of these dominant FAs.
However, the extraction effectiveness on most long chain unsaturated FAs, was significantly dependent on the extraction method \( (P < 0.05, \text{Table 2}) \). When using ScCO\(_2\) for extraction, the yield for each FA was also almost ranked highest. The basic principal of this technology is achieving a certain phase (supercritical) that is beyond the critical point of a fluid, in which the meniscus separating the liquid and vapour phases disappears, leaving only a single homogeneous phase \[35\]. Consequently, the changes of the thermophysical properties transform the fluid into a super-solvent and thus, could improve extraction and reaction efficiency \[34\]. Moreover, ScCO\(_2\) likely shows a better performance on unsaturated FA extraction, demonstrated by its relatively lower proportion of SFA (44.36% of total FA). This is coincident with previous reports that there is a low risk for lipid oxidation or thermal degradation during ScCO\(_2\) extraction \[16-18\].

With this regard, ScCO\(_2\) extraction would be more meaningful for high value FAs studies in microalgae.

Similar to the ScCO\(_2\) extraction, a small amount of DHA was also observed in the Pro:Hex method (Table 2). It cannot be ruled out that this stems from the contribution of both thermal bath and ultrasonication treatments during the Pro:Hex extraction (Figure 3). As a benchmark commonly used for lipid extraction, the Bligh & Dyer method (Chl:Met) was not successful with for extraction of C22:5 (n-3) and C22:6 (n-3) (DHA). This was also not doable when using Dic:Met or saponification (Eth:KOH). Furthermore, the amount of other long chain FAs (e.g., C18:3 (n-3), C20:4 (n-6) and C20:5 (n-3) which are linoleic acid (ALA), eicosatetraenoic acid (ETA) and eicosapentaenoic acid (EPA)) was also significantly different for these extractions \( (P < 0.05, \text{Table 2}) \). At this point, the feasibility of Dic:Met and/or Pro:Hex as an alternative for Chl:Met as suggested above, would be worth considering for lipid profile analyses in microalgae.

| Table 2 Comparison of normalised fatty acids (FA) composition between different extraction methods (% of dry weight) determined from FAME analysis by GC/MS |
|-----------------|------|------|------|------|------|
| MW | Chl:Met | Dic:Met | Pro:Hex | Eth:KOH | ScCO\(_2\) |
| C14 | 242 | 0.03 ± 0.01 (a) | 0.06 ± 0.01 (ab) | 0.12 ± 0.03 (b) | 0.06 ± 0.02 (ab) | 0.19 ± 0.01 (c) |
| C16 | 270 | 3.57 ± 0.14 | 3.63 ± 0.37 | 3.27 ± 0.44 | 3.31 ± 0.32 | 3.81 ± 0.47 |
| C16:1 (n-7) | 268 | 0.14 ± 0.08 (a) | 0.06 ± 0.01 (a) | 0.09 ± 0.02 (a) | – (b) | 0.09 ± 0.03 (a) |
| C16:1 | 268 | 0.39 ± 0.03 (a) | 0.59 ± 0.05 (ab) | 0.58 ± 0.12 (ab) | 0.37 ± 0.06 (a) | 0.81 ± 0.08 (b) |
| C16:2 | 266 | 0.06 ± 0.02 (a) | 0.07 ± 0.01 (a) | 0.12 ± 0.03 (a) | – (b) | 0.21 ± 0.00 (c) |
| C16:3 | 264 | 0.44 ± 0.05 | 0.36 ± 0.06 | 0.38 ± 0.11 | 0.35 ± 0.05 | 0.31 ± 0.02 |
| C18 | 298 | 0.44 ± 0.04 (a) | 0.27 ± 0.01 (b) | 0.43 ± 0.05 (a) | 0.34 ± 0.02 (ab) | 0.38 ± 0.02 (ab) |
| C18:1 (n-9c) | 296 | 0.98 ± 0.47 | 1.26 ± 0.13 | 1.17 ± 0.08 | 0.89 ± 0.07 | 1.25 ± 0.53 |
| C18:1 (n-9t) | 296 | – (a) | 0.06 ± 0.03 (a) | 0.06 ± 0.01 (a) | – (a) | 0.16 ± 0.02 (b) |
| C18:2 | 294 | 0.72 ± 0.07 | 0.52 ± 0.12 | 0.62 ± 0.07 | 0.68 ± 0.13 | 0.73 ± 0.18 |
| C18:3 (n-6) | 292 | 0.15 ± 0.03 (a) | 0.16 ± 0.01 (a) | 0.04 ± 0.01 (b) | 0.05 ± 0.01 (b) | 0.12 ± 0.03 (a) |
| C18:3 (n-3) | 292 | 0.25 ± 0.02 (ab) | 0.43 ± 0.04 (c) | 0.47 ± 0.05 (c) | 0.24 ± 0.07 (a) | 0.41 ± 0.05 (bc) |
| C20 | 326 | 0.03 ± 0.02 | – (a) | – (a) | 0.06 ± 0.04 | 0.04 ± 0.05 |
| C20:1 (n-9) | 324 | 0.50 ± 0.06 (ab) | 0.36 ± 0.03 (a) | 0.47 ± 0.04 (ab) | 0.37 ± 0.02 (a) | 0.60 ± 0.09 (b) |
| C20:4 (n-6) | 318 | 0.12 ± 0.00 (a) | 0.20 ± 0.03 (b) | 0.06 ± 0.02 (a) | 0.12 ± 0.02 (a) | 0.27 ± 0.03 (b) |
| C20:5 (n-3) | 316 | 0.47 ± 0.08 (ab) | 0.32 ± 0.04 (abc) | 0.27 ± 0.05 (bc) | 0.21 ± 0.07 (c) | 0.52 ± 0.06 (a) |
| C22:5 (n-3) | 344 | – (a) | – (a) | – (a) | – (a) | 0.03 ± 0.01 (b) |
| C22:6 (n-3) | 342 | – (a) | – (a) | 0.04 ± 0.00 (b) | – (a) | 0.04 ± 0.00 (b) |
| Total Saturated FA (mean% of total FA) | | 48.65 | 47.43 | 46.98 | 46.83 | 44.36 |
| Total Monounsaturated (mean% of total FA) | | 25.21 | 27.76 | 28.66 | 27.01 | 29.17 |
| Total Polyunsaturated (mean% of total FA) | | 26.14 | 24.81 | 24.36 | 26.16 | 26.47 |
| Total FA (mean% of dry weight) | | 8.33 ± 0.30 (a) | 8.64 ± 0.49 (ab) | 8.18 ± 0.51 (a) | 6.06 ± 0.44 (c) | 10.00 ± 0.27 (b) |

Chl:Met – chloroform and methanol method; Dic:Met – dichloromethane and methanol method; Pro:Hex – propan-2-ol and cyclohexane method; Eth:KOH – ethanol and KOH method; ScCO\(_2\) – supercritical-CO\(_2\) extraction method.

Notes: Values less than 0.03% were deleted from the calculation to eliminate the effect of background and labelled as “–” in the table. The Bold and Italic parameters in the first column were dependent on extraction method, indicated by different small letters in brackets which show significant differences between extractions methods \( (P < 0.05) \).
Conclusion

Through comparison of extraction methods, this study highlights the bias on microalgal lipid recovery, demonstrated by clear differences in microalgal lipid production and FAME profile analyses. As a consequence, different lipid extraction methods selected for microalgal lipid studies can result in widely varying estimations of the lipid-based bioproducts of microalgae. As outlined in previous studies [4,26], the lipid profile and production yields are also highly dependent on microalgal cultivation conditions, biomass processing, cell disruption and strain selection in addition to solvent polarity and extraction processing.

Figure 2 Total amount of saturated, mono- and polyunsaturated fatty acids in microalgal dry biomass (%) across different extraction methods. Chl:Met – chloroform and methanol method; Dic:Met – dichloromethane and methanol method; Pro:Hex – propan-2-ol and cyclohexane method; Eth:KOH – ethanol and KOH method; ScCO2 – supercritical-CO2 extraction method. Different symbols, small and capital letters represent significant differences on saturated, mono- and polyunsaturated fatty acids, respectively, for the different extraction methods ($P < 0.05$).

Figure 3 Brief overview of lipid extraction methods used.
such a scenario, meaningful data for microalgae under consideration for high value products and biodiesel production will require careful choice and validation of analysis methodology. In the present comparison, this study would highly recommend the supercritical CO$_2$ technique for lipid extraction, aiming for an accurate evaluation on the potential of microalgae for high value FA production. Meanwhile, this study also can serve as model for how such studies would be conducted across algal genera that produce triglycerides as their main biodiesel feedstock. From a commercial perspective, a techno-economic assessment is needed and should ideally be carried out for large-scale extraction where costs are likely to be very different compared to the present laboratory-based study.

Methods

Experimental microalgae

Marine microalga *Tetraselmis* sp. (strain M8) was isolated from the Sunshine Coast, Queensland, Australia (26°39′39″S, 153°6′18″E; Genbank accession number JQ423158). By using a 2 × 1,000 L split microalgal cultivation system M8 culture was scaled up and induced for lipid accumulation by nutrient deprivation as described previously [36]. The freeze-dried biomass was ground into a fine powder for subsequent extractions.

In order to improve our understanding and to highlight the importance of extraction method selection, microalgal oil extraction was conducted with two *modi operandi*. First, Soxhlet extraction was performed with either single solvents or a mixture of solvents, and conventional gravimetric methods along with fatty acids analysis through GC-MS were used for quantification and qualification of lipid extraction. The other approach entailed comparative extractions by five different methods, coupled with a technique of Thermo Gravity Analysis (TGA) for microalgal lipid content determination. All solvents used for lipid extractions were HPLC grade.

**Soxhlet extraction: single solvent vs. mixture**

The Soxhlet extraction was implemented with 2 g of lyophilised *Tetraselmis* sp. M8 biomass powder on a Soxhlet system HT (Foss Soxtec 1043): 6 hours of extraction process at 140°C, followed by 30 min solvent rinse and 30 min solvent evaporation. There were two extraction solvent schemes for lipid recovery: 52 ml hexane alone and the mixture (39 ml hexane: 13 ml ethanol) (n = 3).

The weight of oily extract was weighed and counted as oil content (% DW) and subsequent fatty acid analyses were carried out by GC-MS.

**Comparison of five lipid extractions**

Comparative lipid extractions were carried out with 200 mg aliquots of microalgal powder by five different approaches (n = 3, Figure 3). The first extraction method was following Bligh & Dyer [23] with minor modifications. Briefly, the algal powder was eluted by 5 ml of chloroform and methanol (1:2, v/v; CHCl$_3$/MeOH) in a capped glass tube, and placed in an Ultrasonic Cleaner (Unisonics N1984) at room temperature. With an interval of one hour, the samples were added with 2 ml CHCl$_3$ and 3.6 ml water, vigorously vortexed and centrifuged at 1,000 × g for 5 min. The organic phase was pipetted into a new glass tube, and replaced by the same amount of CHCl$_3$ to maintain the extraction volume in the extraction tube for re-extraction. About 4 hours later (4 rinses) when there was no colour appearing in the freshly-added solvent, all organic layers were pooled together and then evaporated using a rotary evaporator (Buchi Rotavapor RE120).

The second extraction method was adopted from Cequier-Sanchez et al. [12]. First, 200 mg of the dry biomass was extracted by immersion in 6–8 ml of dichloromethane-methanol (2:1, v/v; CH$_2$Cl$_2$/MeOH) contained in a capped glass test tube, performing occasional gentle hand agitation for 2 hours. Subsequently, the samples were filtered through a glass fibre filter paper under vacuum and transferred to a new test tube. A total of 1.25 ml of KCl aqueous solution (0.88%, w/v) was added into the filtration, followed by strong agitation and centrifugation at 1,500 × g for 4°C for 5 min. The aqueous phase was discarded, whilst the organic phase was collected for rotary evaporation.

The third method was using propan-2-ol and cyclohexane (1:1.25, v/v; C$_3$H$_6$O/C$_6$H$_{12}$) as described by Schlechtriem et al. [24]. The samples were put into the test tubes and mixed with 9 ml of C$_3$H$_6$O/C$_6$H$_{12}$ followed by 30 s vortexing. Then, the tubes were ultrasonicated at 60°C for 30 min (Unisonics Australia). Then, 5.5 ml of water was added to obtain a mixture with C$_3$H$_6$O/C$_6$H$_{12}$. After 30 s of vortexing, the different phases were separated by centrifugation at 1800 × g for 10 min. When the organic phase was transferred to a new test tube, the sample was extracted again with adding 5 ml of C$_3$H$_6$O/C$_6$H$_{12}$. Such a repeated extraction was ceased after the fifth time when the extract colour became invisible in the organic phase. Similar to the first extraction method, all the organic phases were pooled together and evaporate-concentrated.

The fourth extraction was conducted by direct saponification, adopted from Burja et al. [7]. Briefly, the samples were immersed in 15.2 ml of 3 mM KOH in 96% ethanol in the test tubes. Then the tubes were vortexed at 60°C for 60 min. Samples were cooled to room temperature and filtered as above. The biomass was washed with 4 ml of ethanol and all the alcoholic solutions (incl. the first filtration) were transferred to a graduated mixing cylinder, and 4 ml of water was added. The unsaponifiables were further extracted by adding 8 ml hexane and gently shaking twice. When the layers were separated, the pH was decreased to 1 by adding HCl/H$_2$O (1:1, v/v) solution. Then, both
saponifiable and unsaponifiable lipids in the top layer were recovered by two rounds of addition of 4 ml hexane and gentle mixing. Then the organic layer was evaporated.

Supercritical-\(\text{CO}_2\) extraction of microalgal lipids was performed with commercial-grade \(\text{CO}_2\) in the supercritical facility within the Australian Institute for Bioengineering and Nanotechnology (AIBN) at The University of Queensland. The algal samples were placed in a small glass tube located in a 60 ml extractor. Typically, extraction was carried out with an initial soaking period of 12 h (15 MPa at 40°C). This was followed by a flushing cycle in which \(\text{CO}_2\) was flowed over the sample at a flow rate of 5 ml/min controlled by an ISCO syringe-pump for 30 min.

All the extracts from above were collected and preserved at 4°C for lipid profiling analysis as below. As microalgae possess a large amount of natural antioxidants, addition of antioxidants was not needed for lipid extraction when short expression times were used [11].

**Quantification of the extract content in microalgal dry biomass**

Posterior to the five parallel lipid extractions, the algal residue (defatted biomass) was collected and lyophilised again (10 h), then analysed on a Thermogravimetric Analyser (TGA/DSC 1 Star e System) \((n = 3)\). The setting was with nitrogen at a flow rate of 50 ml min\(^{-1}\), at a programmed heating interval of 10°C min\(^{-1}\), until reaching 550°C. According to our preliminary study, the algal biomass reduction that occurred between 190 and 540°C represented the major difference between algal cells and extracted algal cells as demonstrated using the *Tetraselmis* sp. M8 sample. The range 190-540°C was therefore selected as the effective temperature range for the extracts in the biomass. The mass loss of water residue in the algal biomass (25-190°C) was then deduced to normalise the microalgal biomass loss in the TGA analysis. The difference between original algal biomass and defatted sample indicated the amount of materials being extracted, based on the formula:

\[
\text{Extract content(%) = } \frac{\text{Biomass reduction (190–540°C, mg)}}{\text{Normalised biomass loss(mg)}} \times 100
\]

**Fatty acid methyl ester (FAME) analyses**

The condensed lipid extracts were hydrolysed and methyl-esterified for FAME analysis by GC-MS [36]. Briefly, 100 \(\mu\)l of extract were mixed with 500 \(\mu\)l of 2% \(\text{H}_2\text{SO}_4\)/methanol solution in a 2 ml eppendorf tube by shaking at 80°C for 2 h. In each sample, 100 \(\mu\)g of heneicosanoic acid (Sigma, USA) was added as an internal standard prior to the reaction. A total of 500 \(\mu\)l of 0.9% (w/v) \(\text{NaCl}\) and 500 \(\mu\)l of hexane was then added to the sample which was subsequently vortexed for 20 s and centrifuged at 16,000 \(\times\) g for 3 min. The hexane layer was pipetted into an autosampler vial for FAME quantification. 1 \(\mu\)l of the hexane layer was injected into an Agilent 6890 gas chromatograph equipped with a 5975 MSD mass spectrometer (Agilent Technologies Australia Pty Ltd; GC/MS), for identification of FAMEs. Separation was achieved on a DB-Wax column (Application note: 5988-5871EN) with a cyanopropyl stationary phase with helium as carrier gas in constant pressure mode. Identification of FAME was based on mass spectral profiles, comparison to standards, and expected retention time from Agilent’s RTL DB-Wax method (Application note: 5988-5871EN). In the end, all FAME data were normalised in percentage of dry weight to allow the comparative analysis between different extractions.

**Data analysis**

The variation of FAs and lipid contents between extraction methods was investigated by one-way ANOVA, with a Least Significant Differences (LSD) procedure for the post-hoc comparisons. A significance level of \(P < 0.05\) was used for all tests.

**Competing interests**

The authors declare no competing interests.

**Authors’ contributions**

YL and FG contributed to the experimental design, data acquisition, troubleshooting, analysis and interpretation of data, as well as drafting the manuscript. KJT and WAG helped in sample preparation and execution of work on TGA and \(\text{ScCO}_2\). All authors contributed in data collection from literature and writing of the manuscript. All authors have read and approved the final manuscript.

**Acknowledgements**

The authors wish to acknowledge research grant support by the Australian Research Council and Meat and Livestock Australia. The funding bodies had no influence in the writing of the manuscript and in the decision to submit the manuscript for publication.

**Author details**

1 School of Agriculture and Food Sciences, The University of Queensland, Brisbane QLD 4072, Australia. 2 School of Marine and Tropical Biology, James Cook University, Townsville City QLD 4811, Australia. 3 Australian Institute for Bioengineering and Nanotechnology and Centre for Advanced Imaging, The University of Queensland, Brisbane QLD 4072, Australia.

**Received: 26 July 2013 Accepted: 19 January 2014 Published: 24 January 2014**

**References**


Submit your next manuscript to BioMed Central
and take full advantage of:

• Convenient online submission
• Thorough peer review
• No space constraints or color figure charges
• Immediate publication on acceptance
• Inclusion in PubMed, CAS, Scopus and Google Scholar
• Research which is freely available for redistribution

Submit your manuscript at www.biomedcentral.com/submit

Cite this article as: Li et al: A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. Microbial Cell Factories 2014 13:14.
Critical analysis of current microalga dewatering techniques

Kalpesh K Sharma†1, Sourabh Garg†1, Yan Li1,2, Ali Malekizadeh1 & Peer M Schenk*1

Oil-accumulating microalgae have the potential to enable large-scale biodiesel production without competing for arable land or biodiverse natural landscapes. However, microalga harvesting/dewatering is a major obstruction to industrial-scale processing for biofuel production. The dilute nature of microalgae in cultivation creates high operational costs for harvesting, thus making microalgal fuel less economical. Within the last decade, significant advances have been made to develop new technologies for dewatering or harvesting of microalgae. The choice of which harvesting technique to apply depends on the microalgae cell size and the desired product. Microalgae dewatering processes can broadly be classified as primary and secondary dewatering. This article provides an overview of current dewatering techniques along with a critical analysis of costs and efficiencies, and provides recommendations towards cost-effective dewatering.

Microalgae have a robust photosynthetic capability for fixing CO₂ and converting solar energy into chemical energy. Moreover, they do not need to compete with arable land and freshwater, and have been considered as one of the most promising feedstocks for biofuels [1,2]. Microalgae are typically 2–50 µm in size with a negative charge on the cell surface [3–5], but some microalgae, under certain conditions, have a larger cell size. In most cases they are motile (i.e., swimming or gliding), such as dinoflagellates or raphid diatoms, and form stable suspensions. Unfortunately, microalgal biomass is fairly dilute in cultures (up to 0.3–0.5 g dry biomass/l), resulting in difficulties in harvesting and dewatering algae cost effectively [6]. Microalgae harvesting can typically make up to 20–30% of the total biomass production cost [7–9]. This makes the harvesting process a major bottleneck, hindering the development of the microalgae industry. To date, there are a multitude of techniques being used for microalgae dewatering, but with low economical feasibility. Based on their large biodiversity, microalgae harvesting processes are to a large extent species specific [10,11]. They are also closely linked to cell density and cultivation conditions [12].

The production of biofuel, such as biodiesel, from microalgae is a multistep process involving cultivation, biomass harvest, lipid extraction and oil conversion. Compared with the other processes, harvesting is arguably still the most critical and challenging stage in microalgae biomass production [4,8,12–15]. When considering commercial-scale processes for dewatering and recovering algal biomass for further downstream processes, a traditional harvesting method may involve up to two steps; the first is known as primary harvesting/bulk harvesting, and the second is known as secondary dewatering/thickening (Figure 1) [8–10,16]. During the primary harvesting process, the microalgae mass ratio to water volume is increased [17]. This step aims to achieve a concentration containing 2–7% total solid matter, from the initial biomass concentration [16]. Secondary dewatering concentrates the biomass up to 15–25%, which when followed by drying, aims to further concentrate the slurry, increasing the total solid matter up to 90–95%. This step is generally a more energy-intensive step than...
primary harvesting. Several techniques for dewatering of microalgal cultures have been developed [36].

This article attempts to provide an overview of these techniques to estimate their efficiencies, and then classify these techniques based on their properties. It also highlights the need for developing hybrid technology. It is desired to optimize microalgal dewatering processes by combining the strengths of several different harvesting techniques.

**Primary harvesting**

Primary harvesting methods reviewed here include *floc*ulation, *flo-tation*, sedimentation and electro-floc*ulation* (Figure 1) [1,6,18].

- **Floc*ulation**

Floc*ulation* is often performed as a pretreatment to increase the particle size before using another method (Table 1). Hence, floc*ulation* is commonly used before secondary dewatering processes to facilitate further steps such as centrifugation or filtration [4,10,19]. In some cases negative charges of microalgal cells inhibit aggregation; therefore, cationic floculants, cationic polymers and metal salts (e.g., ferric chloride, alum, aluminum sulfate and ferric sulfate) are used to neutralize charges and facilitate aggregation [4,13,16,19–22]. The efficiency of electrolytes to induce coagulation is measured by the critical coagulation concentration, or the concentration required to cause rapid coagulation. Coagulation efficiency of metal ions increases with increasing ionic charge. Multivalent metal salts such as alum have been widely used to floc*ulate* algal biomass in wastewater treatment processes [23,24]. Alum is an effective floc*ulator* for freshwater species such as *Scenedesmus* and *Chlorella* [25]; however, for maximizing the economic value derived from the feedstock, there is a need to produce various co-products such as pigments, protein, omega-3 fatty acids and animal feed along with biofuel production [26]. Hence, floc*ulation* by metal salts may be unacceptable if biomass is to be used in certain aquaculture applications or to be used as food or feed. Polyferric sulphates are reported to be a better floculant compared with the more traditional nonpolymerized metal salt floc*ulants*, as shown by Jiang *et al.* [27]. Prepolymerized metal salts are effective over a wider pH range than nonpolymerized salts [27]. Moreover, floc*ulation* was carried out by adjustment of pH using sodium hydroxide and addition of the nonanionic polymer Magnafloc LT-25 to a final concentration of 0.5 mg l⁻¹ by Knuc*key et al.* [28].

Ultrasound has also been used to induce aggregation in microalga*e* [29]. Microbial floc*ulation* under nutrient-depletion stress has been investigated by Lee *et al.* [30]. Floc*ulation* occurs naturally in some microalga*e*; for example, by high light, nitrogen stress, and changes in pH, salinity or the level of dissolved oxygen [22]. This typically leads to floc*ulation* and settling, and probably presents a protective survival mechanism for alga*e* in their natural environment.

Electrolytes and synthetic polymers are typically added to coagulate (neutralize charge) and floc*ulate* the cells, respectively [31]. Smith and Davis recently investigated autofloc*ulation* using magnesium-based floc*ulants* naturally available in brackish water [32]. Moreover, magnesium-based floc*ulants* can be obtained from wastewater treatment plants. A recent study carried out by Taylor *et al.* on *Nannochloropsis oculata* observed that artificially treating the algae with algal extracts does not only effectively floc*ulate* microalga*e*, but also increase overall lipid content [33]. Interrupting CO₂ to algal culture may also cause autofloc*ulation* [34,35]. However, autofloc*ulation* may not be as reliable as chemical floc*ulation* [22]. Electrocoagulation floc*ulation* (ECF) has been evaluated as a method for harvesting a freshwater (*Chlorella vulgaris*) and a marine (*Phaeodactylum trico-rnatum*) microalgal species by Vandamme *et al.* [36]. In this study, ECF was shown to be more efficient using an aluminium anode than using an iron anode. Moreover, the efficiency of the ECF process could be substantially improved by reducing the initial pH and by increasing the turbulence in the microalgal suspension. In another study conducted by Xu *et al.*, a rapid and efficient electro-floc*ulation* method integrated with dispersed air floc*ulation* was developed for harvesting *Botryococcus braunii* with a recovery of 98.6% within 14 min [37].

When considering downstream processes to produce bioproducts from algae, the use of metal salts for coagulation and floc*ulation* poses many challenges. In wastewater sludge treatment, aluminium and sulfate have been shown to affect the specific methanogenic activity of methanogenic and acetogenic bacteria, and reduce their anaerobic digestion ability [38]. A similar problem may be faced when using algal biomass for anaerobic digestion. Land application of aluminium-treated sludge can increase heavy metal uptake and cause phosphorus deficiency in plants [39].

Natural polymers that do not raise environmental concerns may also be used as floc*ulants*, although these are less studied. One of the most widely used and studied natural polymers for floc*ulation* is chitosan (at a pH of ~7), which is typically derived from crab shell. Divakaran *et al.* reported successful floc*ulation* and settling of algae by adding chitosan [40], which is considered an environmentally friendly option that has
also been described in various other studies [41–43]. In other studies, nonconventional flocculants such as *Moringa oleifera* seed flour have been used by Teixeira *et al.* as another nontoxic microalgae flocculant [44]. Cationic starch is also mentioned as another potential effective flocculant for freshwater microalgae by Vandalme *et al.* [45].

### Gravity-assisted sedimentation

This process is commonly used in wastewater treatment. However, this process is also appropriate for microalgae larger than 70 µm in size [16,46], but is typically fairly slow due to the low specific gravity of algal cells [4].

### Flotation

In this process, microalgal cells are trapped on microair bubbles and float to the surface [16]. Efficient flotation relies on successful collision and attachment of bubbles and particles, and works best when algal cells are hydrophobic [3,47].

Dissolved air flotation (DAF) has been successfully used in water treatment plants and is also widely used for microalgae harvesting [Table 2] [48,49]. It involves the release of pressurized water (saturated with air) into the tank containing microalgae. Due to the difference of pressure, many fine bubbles form, carrying algal cells as a froth, which can be skimmed off. The effectiveness of this process depends on air bubble size, solubility and the pressure difference of air, the hydraulic retention time, and the floated particle size [50]. Before algae can be removed using DAF they need to be flocculated. The flocculation increases the efficiency of removal. A study carried out by Edzwald found DAF to be more effective than sedimentation [50]. Suspended air flotation is an alternative method that could potentially harvest microalgae with a lower air:solids ratio, lower energy requirements and higher loading rates compared with DAF [51].

In dispersed air flotation (or foam flotation), algal cells are floated in a mechanical cell with a high-speed agitator through which a constant stream of air is passed [3]. Fine bubbles of approximately 1 mm diameter are generated by either ‘agitation combined with air injection’ or ‘bubbling air through porous media’ [52]. Hydrophobic interaction plays an important role for attachment particles, such as microalgae, to the bubbles [3]. Bubbles then rise to the surface and constantly accumulate as foam as a result of solid–liquid separation [3]. Foam fractionation is considered as an alternative to the use of expensive centrifugation for microalgae harvesting [53].

### Secondary dewatering

In secondary dewatering or thickening, the algae slurry is concentrated approximately 10–30-times, and consequently the water content of the produced algae paste can be as low as 20–25% (Figure 1) [13]. Energy-intensive processes such as centrifugation and ultrasonic aggregation are commonly used at this stage [16]. This step requires more energy input than primary dewatering, and therefore needs more capital and operating costs [46].

### Centrifugation

Centrifugation is the ideal method for rapid harvesting of algae containing high-value products. Generally centrifuges can be of various types and sizes depending on the uses. A disc stack centrifuge consists of a relatively shallow cylindrical bowl containing a number (stack) of closely spaced metal cones (discs) that rotate, and it is mostly used in commercial plants for high-value algal products and in algal biofuel pilot plants. Decanter centrifuges have been found to be as effective as solid-bowl centrifuges for separating microalgae, but the energy consumption of decanter centrifuges is higher than that of disc-bowl centrifuges at 8 kWh m⁻¹ [8]. A hydrocyclone is a relatively low-energy (0.3 kWh m⁻¹) particle-sorting device compared with other centrifuge methods, but on the other hand it was reported...
to be an unreliable means of concentrating microalgae as only a maximum concentration factor of 4 could be achieved [8]. Spiral plate centrifuges are considered a relatively new generation of centrifuges, manufactured by Evodus. The suspension flows outwards in thin films over vertical plates with the solid sediment or microalgae being forced by centrifugal force to collect on the outer bottom edge of the vanes. Table 3 provides more analyses and details about the harvesting of 10,000 l of *Chlorella* sp. with an Evodos centrifuge. More detailed studies on centrifuge harvesting have been carried out by Molina Grima *et al.* [8]. However, centrifugation is energy intensive, not easily scalable and requires high maintenance due to fast-moving mechanical parts [8,16,22]. Therefore, centrifugation has high capital and operating costs, and is considered too expensive for low-value products such as biofuel [8,54]. Furthermore, high speed spinning can disrupt algae cells [19,55].

Table 2. Examples of various flotation studies that have been used to harvest microalgae.

<table>
<thead>
<tr>
<th>Algae</th>
<th>Surfactant</th>
<th>Surfactant type</th>
<th>Ph</th>
<th>Dosage (mg l⁻¹)</th>
<th>Result</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Scenedesmus quadricauda</em></td>
<td>SDS + chitosan</td>
<td>Anionic surfactant</td>
<td>8.0</td>
<td>20 + 10</td>
<td>95% in 20 min</td>
<td>[82]</td>
</tr>
<tr>
<td><em>Chlorella</em> sp.</td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>8.0</td>
<td>40</td>
<td>86% in 20 min</td>
<td>[83]</td>
</tr>
<tr>
<td><em>Scenedesmus quadricauda</em></td>
<td>SDS + chitosan</td>
<td>Anionic surfactant</td>
<td>8.0</td>
<td>20 + 10</td>
<td>85–90% in 20 min</td>
<td>[83]</td>
</tr>
<tr>
<td><em>Chlorella</em> sp.</td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>7.8</td>
<td>100</td>
<td>&gt;90% in 20 min</td>
<td>[84]</td>
</tr>
<tr>
<td><em>Chlorella</em> sp.</td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>9.5</td>
<td>1–3</td>
<td>95–99% in 11 min</td>
<td>[3]</td>
</tr>
<tr>
<td><em>Chlorella</em> sp.</td>
<td>CTAB</td>
<td>Cationic surfactant</td>
<td>10</td>
<td>45</td>
<td></td>
<td>[85]</td>
</tr>
<tr>
<td><em>Dunaliella salina</em></td>
<td>Aluminium sulphate</td>
<td>Inorganic metallic coagulants</td>
<td>5</td>
<td>150</td>
<td>95% in 30 min</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td>Ferric sulphate</td>
<td></td>
<td>5</td>
<td>150</td>
<td>98% in 30 min</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ferric chloride</td>
<td></td>
<td>5</td>
<td>75</td>
<td>98.7% in 30 min</td>
<td></td>
</tr>
</tbody>
</table>

CTAB: Cetyl trimethyl ammonium bromide; SDS: Sodium dodecyl sulfate.
Critical analysis of current microalgae dewatering techniques

Filtration

Filtration methods such as microstrainers, vibrating screen filters, and micro- and ultra-filtration have been widely studied, and have proven to be efficient (Table 4) [56–58]. One of the major disadvantages of these techniques is the high capital and operating costs to avoid filter blinding and disruptive pressure changes (high pressure or vacuum). Membrane filtration and ultrafiltration are costly for large-scale operations due to high operating costs for membrane replacement, clogging and pumping [4,8,10,13,16,22,46,57]. Although the filtration process may be considered slower than centrifugation for some applications [10], it is still a simpler and lower cost alternative when compared with centrifugation, if implemented properly. Fast formation of thick filter cake, which dramatically decreases flow rate, is another disadvantage of conventional filtration processes [22].

Cross-flow filtration (tangential flow filtration) has been shown to solve these problems as the filter cake is washed away during the cross-flow filtration process, which increases the operation time of the filtration system [59]. However, this technology is still very expensive for low-value products and is not easily scalable. In addition, most studies consider the conventional filtration process as unsuitable for harvesting of small microalgae (smaller than 30 µm) [4,10,13,16,22,60].

Drying

The water content of algal paste after secondary dewatering should not exceed 50% before oil extraction [12]. Because the cost of thermal drying is high (even higher than mechanical drying), a harvesting method with a high solid content is preferable before drying [10]. Common methods for drying microalgae after secondary dewatering are: spray drying, drum drying, freeze drying and sun drying [10]. Spray drying is considered too expensive for low-value products such as biofuel [10]. The influence of short-term storage and spray and freeze drying of fresh microalgal paste on the stability of lipids and carotenoids of *P. tricornutum* was investigated by Ryckebosch et al. [61]. Solar drying is considered the most economical drying process; however, it requires large land areas for large-scale operations [12,62].

Technoeconomic assessment

Using the information from previously completed studies and specifications provided by companies that supply the equipment and chemicals, a theoretical calculation

### Table 3. Cost of harvesting 10,000 l of *Chlorella* sp. with different harvesting techniques.

<table>
<thead>
<tr>
<th></th>
<th>Single step</th>
<th>Primary</th>
<th>Secondary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Centrifugation</td>
<td>Sedimentation</td>
<td>Flotation (1)</td>
</tr>
<tr>
<td>Total energy consumed (kWh/10 m³)</td>
<td>55¹</td>
<td>–</td>
<td>7.4–8.4³</td>
</tr>
<tr>
<td>Energy cost (AUS)</td>
<td>12.10±⁴</td>
<td>–</td>
<td>1.62–1.84⁴</td>
</tr>
<tr>
<td>Dosage required (g)</td>
<td>–</td>
<td>100 @ 10 mg/l [1]</td>
<td>30 @ 3 mg/l [1]</td>
</tr>
<tr>
<td>Chemical cost (AUS)</td>
<td>–</td>
<td>2.50 (chitosan @ $25/kg)</td>
<td>0.24 (CTAB @ $8/kg)</td>
</tr>
<tr>
<td>pH adjustment dosage</td>
<td>–</td>
<td>1.5–2 l acetic acid††</td>
<td>–</td>
</tr>
<tr>
<td>pH adjustment cost (AUS)</td>
<td>–</td>
<td>1.20–1.60 @ $800/ton</td>
<td>–</td>
</tr>
<tr>
<td>Total cost (AUS)</td>
<td>12.10</td>
<td>3.70–4.10</td>
<td>1.86–2.08</td>
</tr>
</tbody>
</table>

¹An Evodos centrifuge was used for this study.
²Flotation cell considered is Jameson cell and energy consumption was determined using various published studies as well as our own published data.
³Flotation cell considered is column flotation cell and energy consumption was determined using work done by Coward et al. [85].
⁴1 AUS = ~US$1.04.
⁵Electricity prices were calculated based on AUS0.22/kWh.
⁶The volume was estimated by doing an experiment with 1 l of algae culture.
CTAB: Cetyl trimethyl ammonium bromide.

### Table 4. Examples of various filtration studies that have been used to harvest microalgae.

<table>
<thead>
<tr>
<th>Species</th>
<th>Type of filtration</th>
<th>Effective (% TSS)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coelastrum sp.</td>
<td>Non-precoated vacuum drum filter</td>
<td>18</td>
<td>[6]</td>
</tr>
<tr>
<td>Coelastrum sp. and</td>
<td>Potato starch vacuum drum filter</td>
<td>37</td>
<td>[6]</td>
</tr>
<tr>
<td>Scenedesmus sp.</td>
<td>Belt filter</td>
<td>9.5</td>
<td>[6]</td>
</tr>
<tr>
<td>Chlorella and Cyclotella</td>
<td>Microfiltration</td>
<td>–</td>
<td>[50]</td>
</tr>
<tr>
<td>Scenedesmus quadricauda</td>
<td>Ultrafiltration membranes</td>
<td>–</td>
<td>[87]</td>
</tr>
<tr>
<td>Spirulina sp.</td>
<td>Ultrafiltration membranes</td>
<td>20</td>
<td>[88]</td>
</tr>
<tr>
<td>Spirulina microactinium</td>
<td>Rotary vacuum filter</td>
<td>1–2</td>
<td>[89]</td>
</tr>
<tr>
<td>Spirulina</td>
<td>Belt filters</td>
<td>18</td>
<td>[8,90]</td>
</tr>
<tr>
<td>Haslea ostrearia</td>
<td>Crossflow microfiltration and ultrafiltration</td>
<td>–</td>
<td>[91]</td>
</tr>
</tbody>
</table>

TSS: Total suspended solids.
was carried out to determine the technoeconomic feasibility of overall biomass recovery in a one-step as well as a two-step method. For the costing purpose, harvesting of 10,000 l of Chlorella sp. culture was considered (Table 3). Assessments were independently developed in accordance with Australian conditions and, where possible, were compared to equivalent costing from previous economic analyses of microalgae biofuel systems.

Table 3 compares some of the traditionally used harvesting methods in microalgae bioprocessing. From the table it can be summarized that due to its high energy consumption, single-step centrifugation is the most expensive method when compared with other techniques.

Flotation appears to be the most cost-effective method for primary dewatering; however, if used with centrifugation, the overall setup costs will increase and would result in higher capital costs. On the other hand, for flotation, if used in conjunction with filtration, the overall process may become more feasible but there is still room for improvement. Moreover, the cetly trimethyl ammonium bromide chemical used for flotation is not only toxic to the environment but also makes the biomass unfit for human and animal consumption. Flocculation coupled with filtration may be more cost effective, but chitosan used for flocculation is biodegradable, as it is derived from a biological source (crustacean). However, large-scale use of chitosan may not be possible as it is expensive, as well as this putting pressure on crustacean populations. Commercial chitosan is derived from the shells of shrimp and other sea crustaceans, including Pandalus borealis. Hence, some harvesting techniques are more feasible than others when considering costs only, but some of these may not be environmentally friendly. Thus, there is a need to optimize current methods or to develop improved methods that are not only cost effective, but also environmentally friendly.

### Classification of current harvesting processes

Current harvesting methods mentioned above can be divided into chemically based, mechanically based and biologically based (Figure 2). Various combinations or sequences of these methods can be used for cost-effective harvesting. Currently, biologically based methods are being investigated as a cost-reducing and environmentally friendly means of harvesting [63]. In any case, it needs to be checked if any desirable valuable compounds are lost during the process. To develop a cost-effective harvesting technique, apart from the costs, one has to consider the following three main aspects: species-specific requirements of microalgae that need to be harvested; recovery/yield of desired product; and environmental impact.

Chemically based methods can be termed as a harvesting method that involves the addition of chemicals to the microalgae culture to induce flocculation, which is used in various solid–liquid separation processes as a pretreatment stage [64]. The chemical reactions are highly sensitive to pH, and high doses of flocculants are required to produce large amounts of sludge, which may leave a residue in the treated effluent. Although cost effective, a major disadvantage could be the presence of harmful salts and chemicals in the extracted biomass, which can possibly pose health and environmental risks. For example, use of aluminium oxide to flocculate microalgae can lead
to accumulation of aluminium salt precipitates in the biomass.

Mechanical harvesting, as the name suggests, is the method that involves the use of a mechanical machine to harvest microalgae, which generally includes centrifugation, filtration and flotation. Molina Grima et al. concluded that centrifugation is a preferred method for harvesting of microalgal biomass, especially for producing extended shelf-life concentrates for aquaculture, pharmaceuticals and other high-value products such as omega-3 [8]. However, Knuckey et al. state that exposure of microalgae cells to high gravitational and shear forces can damage the cell structure [28]. In addition, processing a large amount of culture using centrifugation is time consuming and increases the overall costs of microalgae biomass production (Table 3). Filtration and gravitational sedimentation are widely applied in wastewater treatment facilities to harvest relatively large (>70 µm) microalgae such as Coelastrum and Spirulina. However, they cannot be used to harvest algae species approaching bacterial dimensions (<30 µm) such as Scenedesmus, Dunaliella and Chlorella, which can rapidly and easily blind the filter [16]. This may result in higher operating costs and frequent replacement of filters. In summary, most technologies including chemical and mechanical methods greatly increase operational costs for algal production and are only economically feasible for production of high-value products [65].

Biological harvesting is the method in which bio-products or other microorganisms are used for the harvesting of microalgae. When cultivating microalgae, some cultures tend to aggregate and grow as fluffy pellets, or tightly packed, compact or dense granules. These fluffy pellets are caused by filamentous microorganisms, including some species of molds and bacteria [66–68], and may assist in trapping additional microalgal cells, one of the major advantages of cell pelletization [7,63,68]. Fungal cell growth can be induced by changing operational conditions during cell cultivation, rather than using CaCO₃ powder or other nuclei to induce the fungal pelletization [66], which are costly and cause solid waste disposal issues. A preliminary study was recently conducted by Zhou et al. to inoculate filamentous fungal spores when culturing mixotrophic green algae, C. vulgaris, with the result that pellets clearly formed within 2 days of culture [68]. Microalgal cells, aggregated together with fungal cells, were immobilized in the pellets [67]. Bioflocculation using flocculating microalgae has also been investigated by others [63,68]. The advantage of this method is that neither addition of chemical flocculants is required nor the cultivation conditions have to be changed. This method is as simple and effective as chemical flocculation; however, it is potentially more sustainable and cost effective. No additional costs are involved for pretreatment of the biomass before oil extraction and for the medium before it can be reused [27]. An interesting method is the use of zooplankton to harvest microalgae [69]. Biological harvesting could be a cost-effective method to harvest microalgae, but it is time consuming and has limitations in large-scale cultivation, as enough bioproduct must be co-produced. In addition, chances of cross-contamination are very high.

Based on our analyses conducted on microalgae harvesting technologies (Table 3), it is evident that harvesting techniques should not only be cost effective and rapid, but also have to be environmentally safe and easily scalable for a microalgae-based biorefinery industry. Thus, there is a need to think outside the box and develop new hybrid methods that may combine the best aspects of several techniques (Figure 2).

- **Biochemically based methods**

As described above, flocculation assisted by chitosan (biologically derived) has been used in many studies on different microalgae and has proven to be very promising [41,70,71]. Another example of biologically derived flocculation is the use of M. oleifera seeds, which have also been used for water treatment due to their high flocculation potential, low cost and low toxicity. Recently, Teixeira et al. demonstrated M. oleifera as a successful flocculating agent for C. vulgaris [44]. In addition, a range of new bioflocculants are proposed to address the cost and environmental concerns for current flocculation methods [14]. Microalgae flocculation was also achieved by using naturally available ions in brackish water, and a variety of precipitating ions, including Mg²⁺, Ca²⁺ and CO₃²⁻, can lead to autoflocculation of microalgae [32]. A combination of bioflocculants together with a low dose of chemicals may lead to the best flocculation outcome.

- **Emerging technologies**

When considering chemical, mechanical and biological harvesting methods, each method has its advantages and disadvantages. Biomechanical and chemical–mechanical methods for flocculation are less explored when compared with other methods. Developing hybrid techniques, which make use of all three harvesting categories, may be a viable option that is worth exploring.

The conceptual photobioreactor shown by Chen et al. has the potential to be developed into a commercially viable microalgae cultivation system with zero electricity consumption [56]. This was made possible by combining sunlight and multi-LED light sources with solar panels and a wind power generator. Similarly, when considering harvesting, electricity cost is the key factor that makes the process costly, but renewable energy sources such as solar and wind can be used to generate green
Perspective  Sharma, Garg, Li, Malekizadeh & Schenk

electricity [72]. The main disadvantage of these systems is the high construction costs.

Another option to reduce the cost of harvesting could be by combining two or more stages of microalgal biodiesel production with a harvesting method into one step; for example, as done in the study carried out by Taylor et al. [33]. By doing so, not only can the cost be reduced, but also the overall time required for a full production cycle. For example, developing a process that can help in rapid induction of lipids as well as flocculation could accelerate the harvesting process. Similarly, a method was developed by Hejazi et al. for milking β-carotene from Dunaliella salina in a two-phase bioreactor [73]. In this technique, cells were first grown under normal growth conditions and then stressed by excess light to produce larger amounts of β-carotene, and later a biocompatible organic phase was added and the β-carotene was extracted selectively via continuous recirculation of a biocompatible organic solvent through the aqueous phase containing the cells. Because the cells continue to produce β-carotene, the extracted product was continuously replaced by newly produced molecules. Therefore, the cells are continuously reused and do not need to be grown again. Thus, in contrast to existing commercial processes, this method does not require harvesting, concentrating and disruption of cells for extraction of the desired product [73,74].

Matrix-attached algae culture systems have been developed for growing microalgae on the surface of polystyrene foam to simplify the cell harvest [75]. These methods are innovative and will decrease the harvesting costs to some extent if developed successfully, but require heavy investments on equipment and chemical supplies with various combinations or sequences of these methods. Xu et al. developed a simple and rapid in situ magnetic harvesting method by using Fe₃O₄ nanoparticles on B. braunii and Chlorella ellipsoidea. Magnetic particles were added to the microalgal culture broth and then separated by an external magnetic field [76]. Recently a genetically modified approach has also been used for harvesting microalgae of genera Chlamydomonas, Dunaliella, Scenedesmus and Hematococcus sp. [76].

Conclusion
When considering the research carried out in the field of harvesting microalgae over the past few decades, much progress has been made. Researchers have optimized various techniques; machines have become more energy efficient. There is a need to optimize current methods or to develop improved techniques that are not only cost effective, but also environmentally friendly. Moreover, there is a need to develop hybrid harvesting technology that can use the best of all current harvesting methods. The costing calculation in this review suggests that flotation for primary dewatering coupled with filtration maybe the most cost-effective method for microalgal harvesting, but this may be different for different microalgal strains. The comparison also highlights the fact that none of the harvesting methods are cost effective when considering cultivation of microalgae solely for biodiesel production. Hence, it is a necessity to derive a secondary product that has a higher market value when compared with biodiesel. In the past, the majority of studies have focused on freshwater microalgae species and not much work has been done on marine species. With limited availability of freshwater, further research should be focused more on the processing of marine microalgae.

Future perspective
Rapid depletion of fossil fuels and rising GHG emissions have made the case of microalgae as a biofuel source even more compelling. Moreover, microalgae grown on nonarable land have great potential for provision of animal feed, and microalgae can also be used for wastewater purification. At present, harvesting technologies are costly and labor intensive, but recent studies indicate that major efforts are underway to develop new, more efficient and cheaper harvesting technologies, many of which will be microalgae strain specific. Microalgae are being grown in outdoor ponds, greenhouses, photobioreactors, fermenters and hybrid systems combining bioreactors and ponds. As more and larger microalgae pilot plants will be in operation within 5–10 years, more accurate economic assessments of different harvesting methods will be possible that will feed into the life cycle analyses of future algal biorefineries. With the availability of new and more efficient harvesting systems, microalgae harvesting will be less costly, easier to manage and more accessible for farmers, rural communities and industry around the world. Microalgal biorefineries are expected to be first established on a large scale in countries with high irradiation, flat, nonarable, desert, saline or low-biodiversity land, and access to water unsuitable for human consumption or irrigation (brackish, marine or polluted).

Financial & competing interests disclosure
The authors wish to thank the Australian Research Council and Meat & Livestock Australia for financial support. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.
Critical analysis of current microalgae dewatering techniques

Executive summary

Background
- Microalgae harvesting/dewatering is a major obstruction to industrial-scale processing of microalgae for biofuel production.
- When considering commercial-scale processes in order to dewater and prepare algal biomass for further downstream processing, a traditional harvesting method may involve up to two steps; the first is known as primary harvesting/bulk harvesting and second is known as secondary dewatering/thickening.

Classification of harvesting techniques
- Primary harvesting includes flocculation, flotation and sedimentation. Secondary harvesting includes centrifugation and filtration.
- Current harvesting methods can be classified into chemically, mechanically and biologically based categories.

Costing & analyses of current harvesting techniques
- Single-step centrifugation is the most expensive method when compared with other techniques.
- Flocculation coupled with filtration may be cost effective, but chemicals used in flocculation of microalgae may lead to environmental damage.
- Flotation could be the most cost-effective method for primary dewatering; however, if used with centrifugation the overall setup costs increase and result in higher capital costs.

Future perspective
- There is a need to think outside the box and develop new hybrid methods that use best-of-all techniques and are not only cost effective, but also have a low environmental impact.

References
Papers of special note have been highlighted as:
- of interest
- of considerable interest

Perspective Sharma, Garg, Li, Malekizadeh & Schenk

39 Bugbee GJ, Frink CR. Alum sludge as a soil amendment: effects on soil properties and plant growth 1985. Connecticut Agricultural Experiment Station, CT, USA (1985).
51 Clearly explains how bubbles and coagulants affect the overall dynamics of flotation. Demonstrates that by varying the bubble size and coagulant concentration, the overall algal biomass recovery can be significantly improved.
Critical analysis of current microalgae dewatering techniques


Reports the use of dispersed air column flotation for harvesting Chlorella sp., achieving a very high (230-fold) concentration. The authors used a very small amount (0.015 kWh/m³ algal culture) of electricity and 10 mg/l of collector chemical (CTAB).


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

David K. Y. Lim¹, Sourabh Garg¹, Matthew Timmins¹,², Eugene S. B. Zhang¹, Skye R. Thomas-Hall¹, Holger Schuhmann¹, Yan Li¹, Peer M. Schenk¹*

¹School of Agriculture and Food Sciences, The University of Queensland, Brisbane, Queensland, Australia, ²ARC Centre of Excellence in Plant Energy Biology, Centre for Metabolomics, School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, Australia

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 biomass (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-add products such as protein, carbohydrates and a range of fatty acids (FAs). High value omega-3 fatty acids (ω-3) such as...
The current cost of producing 1 tonne of microalgal biomass with are currently hindering economic viability. It was estimated that unfortunately offset by current limitations to economically produce production viable. To produce value-adding products in addition to biodiesel is important to reduce production cost and make large-scale production viable.

The inherent advantages of a microalgal fuel source are unfortunately offset by current limitations to economically produce it on a large-scale. For example, the cost for obtaining dry biomass, large hexane requirements and limited hexane recycling capacity are currently hindering economic viability. It was estimated that the current cost of producing 1 tonne of microalgal biomass with an average 55% (w/w) oil content needs to be reduced by 10-fold in order to be competitive with petroleum diesel [8]. Furthermore, despite estimates that suggest microalgal oil production (US$9–$25/gallon in ponds, $15–$40 in photobioreactors) could be cheaper than the current price of oil [20], companies commercially producing microalgae have not been able to achieve the predicted yields and production costs. Typical lipid yields of 10 g m⁻² d⁻¹ (Skye Thomas-Hall, personal communication) are still short of achieving the current best case scenarios of 103 to 134 g m⁻² d⁻¹ [21]. The industry is still in its infancy, although recent research and development efforts by large oil companies (e.g. Exxon, BP, Chevron and Shell) would certainly increase production capacity and decrease production costs.

As large variations (10–50%) in lipid content exist between different species of microalgae [22,23], it is necessary to identify strains with high lipid content and suitable lipid composition. The need for high-yielding microalgae is straightforward, as this directly translates to an overall increase in production, although lipid production during normal growth needs to be distinguished from lipid accumulation in response to adverse conditions (e.g. nutrient starvation). Lipid composition is equally important, as quantitative and qualitative differences in the TAG content of a given species will affect the quality of biodiesel and its ability to meet fuel standards. Fuels with high cetane number fatty acids (e.g. myristic acid, palmitic acid, stearic acid) are desirable [24], as higher cetane fuels have better combustion quality and the right cetane number of biodiesel is required to meet an engine's cetane rating [25]. Microalgal lipids are mostly polysaturated, which have a low cetane number and are more prone to oxidation. This can create storage problems and are thus preferred to be at a minimum level for biodiesel production. Nevertheless, polysaturated fatty acids lower the cold filter plugging point (CFPP) of fuel and are crucial in colder climates to enable the biodiesel to perform at lower temperatures [3]. With these factors in mind, an “ideal composition” of fatty acids would consist of a mix of saturated and monounsaturated short chain fatty acids in order to have a very low oxidative potential whilst retaining a good CFPP rating and cetane number.

To date, research efforts have focused on lipid production of individual species, usually investigating the effects different growth conditions have on lipid production and content [26,27,28,29,30]. Unfortunately, direct comparisons of results between studies are unreliable, given the different growth conditions and experimental parameters of each species and also the different methods used for lipid extraction. There is growing interest to compare lipid content and FA composition of multiple microalgae species [11,31,32,33,34,35]. Several studies have revealed algae genera such as Tetraselmis, Nannochloropsis and Isochrysis to have highest high lipid content, particularly under nutrient-deprived conditions [11,31].

Nutrient deprivation is regarded as an efficient way to stimulate lipid production in microalgae in several microalgae species [11,29,36,37], especially saturated and monosaturated FAs [6,38,39]. Unfortunately, lipid accumulation is often associated with a reduction in biomass, which reduces overall lipid accumulation. A batch culture strategy can be adopted to obtain maximal biomass productivity as well as induction of lipid accumulation through nutrient deprivation. Although a common research practice, only Rodolfi et al. [11] have published lipid profiles of multiple microalgae species in a batch culture setting.

The target of our work was to identify the most effective microalgae strains for biodiesel production using a basic batch culture strategy. Most studies utilize experimental designs that include aeration of media volumes of 1 L to 10 L in order to identify microalgae strains with high lipid content [31,32,33,36,40]. To provide a direct comparison between different species, this study evaluated eleven microalgae strains collected from local Australian coastal waterways and other collections that originate in various places in the world. Strains were first characterized by microscopy and partial 18S ribosomal RNA sequencing and total fatty acid methyl ester (FAME) contents were then analyzed via GC/MS, which quantifies the fatty acids in triacylglycerides in each strain, thus providing the most accurate representation of the substrate available for biodiesel production. Using growth rate, FAME productivity and FA composition as criteria, this study identified several algae strains to be suitable for biodiesel, including Tetraselmis sp. and Nannochloropsis sp. as highly versatile candidate strains for a multiple-product algal biorefinery.

Materials and Methods

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°31’1”S 153°0’32”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 algae-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.
Table 1. Sources and 18S rRNA sequence accessions of microalgal strains used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Genbank Accession</th>
<th>Location of Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetraselmis sp. M8</td>
<td>JQ423158</td>
<td>Maroochydore, Qld, Australia</td>
</tr>
<tr>
<td>Tetraselmis chai</td>
<td>JQ423150</td>
<td>East Lagoon, Galveston, TX, USA</td>
</tr>
<tr>
<td>Tetraselmis suecica</td>
<td>JQ423151</td>
<td>Brest, France</td>
</tr>
<tr>
<td>Nanochloropsis sp. BR2</td>
<td>JQ423160</td>
<td>Brisbane River, Brisbane, Australia</td>
</tr>
<tr>
<td>Dunaliella salina</td>
<td>JQ423154</td>
<td>Alice Springs, NT, Australia</td>
</tr>
<tr>
<td>Chaetoceros calcitrans</td>
<td>JQ423152</td>
<td>Unknown</td>
</tr>
<tr>
<td>Chaetoceros. muelleri</td>
<td>JQ423153</td>
<td>Oceanic Institute, Hawaii, USA</td>
</tr>
<tr>
<td>Pavlova salina</td>
<td>JQ423155</td>
<td>Sargasso Sea</td>
</tr>
<tr>
<td>Pavlova lutheri</td>
<td>JQ423159</td>
<td>Unknown location, UK</td>
</tr>
<tr>
<td>Isochrysis galbana</td>
<td>JQ423157</td>
<td>Unknown location, UK</td>
</tr>
<tr>
<td>Chlorella sp. BR2</td>
<td>JQ423156</td>
<td>Brisbane River, Brisbane, Australia</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0040751.t001

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% change in cell density. A total of 1 mL of pre-culture in late-log phase was used as inoculum (7 days 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 x 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 x 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 heicosenoic 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 x 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’-GCCTAATTCCACGCTCGAATGC-3’ and Reverse: 5’-GACCATCTCCCCTCCCCGAACC-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 algae 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 Tetraselmis 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 MAFIT [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 Ny and Nx being the number of cells at the start (tx) and end (ty) of the growth phase (7 days). Average doubling time (TAve) 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 ($\mu_{Max}$) was calculated between the 2 days of maximum slope on the average cell density x-axis time plot [31,46]. Lipid productivity ($\mu$ mL $^{-1}$ day $^{-1}$) was calculated as total lipid content ($\mu$g/mL) over the duration of the entire batch culture (laboratory cultures – 9 days, outdoor culture – 12 days).
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 1.3×10^6/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 dependant 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/QST) 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 until 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} day^{-1}) and P. salina (0.45 μL^{-1} day^{-1}) (Table 2), that were significantly (p<0.05) higher to all other species that had a μave of 0.34 μL^{-1} day^{-1}. Specific growth rates (μexp) were also compared with ANOVA, revealing that T. chui had the highest μexp at 1.03 μL^{-1} day^{-1}, followed by Tetraselmis sp. M8 (0.95 μL^{-1} day^{-1}) and P. salina (0.88 μL^{-1} day^{-1}). The fastest doubling times that were significantly different to the others were found for P. lutheri (1.45 days) and Tetraselmis sp. M8 (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. (0.24 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, 3.9 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 mostly not C18:3 (28.9%) and C16 (22.5%), as well as C18:2s (11.7%). D. salina and Chlorella sp. BR2’s FA profile consisted mostly (nearly 90%) of C16, C18 and their unsaturated derivatives. In T. chui and T. suecica, C16 (35–37%), unsaturated C18s (37–43%) and unsaturated C20s (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...
Table 2. Growth rate analysis of eleven microalgae strains during growth phase (7 days) of batch culture.

<table>
<thead>
<tr>
<th>Species</th>
<th>$\bar{P}_{\text{Ave}}$</th>
<th>$\bar{P}_{\text{Exp}}$</th>
<th>Day of $\bar{P}_{\text{Exp}}$</th>
<th>DT $\bar{P}_{\text{Ave}}$ [days]</th>
<th>Cell density $\bar{Y}_{\text{Ave}}$ [$\times 10^6$ cells mL$^{-1}$]</th>
<th>Dry weight (g L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nannochloropsis</em> sp. BR2</td>
<td>0.32</td>
<td>0.62$^{c, d}$</td>
<td>2–4</td>
<td>2.18$^a$</td>
<td>48.4</td>
<td>0.53</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp. M8</td>
<td>0.35</td>
<td>0.93$^{a, b}$</td>
<td>2–4</td>
<td>2.00$^b$</td>
<td>2.07</td>
<td>0.75</td>
</tr>
<tr>
<td><em>T. chui</em></td>
<td>0.35</td>
<td>1.03$^a$</td>
<td>2–4</td>
<td>1.98$^a$</td>
<td>1.56</td>
<td>0.42</td>
</tr>
<tr>
<td><em>T. suecica</em></td>
<td>0.37</td>
<td>0.5$^d$</td>
<td>0–2</td>
<td>1.85$^b, c$</td>
<td>1.52</td>
<td>0.73</td>
</tr>
<tr>
<td><em>D. salina</em></td>
<td>0.30</td>
<td>0.76$^{b, c, d}$</td>
<td>2–4</td>
<td>2.31$^d$</td>
<td>2.14</td>
<td>0.37</td>
</tr>
<tr>
<td><em>C. calcitrans</em></td>
<td>0.34</td>
<td>0.59$^{a, b}$</td>
<td>0–2</td>
<td>2.03$^c$</td>
<td>4.71</td>
<td>n/a</td>
</tr>
<tr>
<td><em>C. muelleri</em></td>
<td>0.35</td>
<td>0.71$^{b, c, d}$</td>
<td>0–2</td>
<td>1.94$^{b, c}$</td>
<td>4.65</td>
<td>0.50</td>
</tr>
<tr>
<td><em>I. galbana</em></td>
<td>0.35</td>
<td>0.61$^{b, c}$</td>
<td>0–2</td>
<td>1.96$^{b, c}$</td>
<td>4.45</td>
<td>0.45</td>
</tr>
<tr>
<td><em>P. lutheri</em></td>
<td>0.48$^a$</td>
<td>0.76$^{b, c, d}$</td>
<td>0–2</td>
<td>1.45$^a$</td>
<td>3.95</td>
<td>0.45</td>
</tr>
<tr>
<td><em>P. salina</em></td>
<td>0.45$^a$</td>
<td>0.88$^{b, c}$</td>
<td>2–4</td>
<td>1.54$^{b}$</td>
<td>5.47</td>
<td>1.68</td>
</tr>
<tr>
<td><em>Chlorella</em> sp. BR2</td>
<td>0.34</td>
<td>0.86$^{b, c}$</td>
<td>0–2</td>
<td>2.05$^c$</td>
<td>13.8</td>
<td>0.59</td>
</tr>
<tr>
<td><em>Tetraselmis</em> sp.M8</td>
<td>0.47</td>
<td>0.48</td>
<td>6–7</td>
<td>1.45$^c$</td>
<td>1.61</td>
<td>0.58</td>
</tr>
</tbody>
</table>

1Value represents mean of two replicate samples.
2Different letter superscripts down a column indicate significant difference at 95% level (ANOVA, Bonferroni’s test; P<0.05).
3Mid-scale outdoor culture.

doi:10.1371/journal.pone.0040751.t002

Evaluation of Oil-Producing Microalgae for Biofuel

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, easy 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 1.6×10^6 cells mL$^{-1}$ on day 7, eventually arriving at 2.3×10^6 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 significant 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 such 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 such 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.

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 such 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.

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 such 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.

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 such 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.

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 such conditions in an outdoor environment. Thus, a locally isolated strain would likely adapt better to local changing environmental conditions and provide a more stable and productive culture.
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
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.
doi:10.1371/journal.pone.0040751.g003

Figure 4. Growth curves of different microalgae in this study. C. calcitrans, C. muelleri, 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)).
doi:10.1371/journal.pone.0040751.g004
l 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. chu 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 Chlorrella 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 Chlorrella 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 Chlorrella 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.*

doi:10.1371/journal.pone.0040751.g005
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).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th><strong>Nannochloropsis sp.</strong></th>
<th><strong>T. chui</strong></th>
<th><strong>T. suecica</strong></th>
<th><strong>Tetraselmis sp. M8</strong></th>
<th><strong>D. salina</strong></th>
<th><strong>C. calcitrans</strong></th>
<th><strong>C. muelleri</strong></th>
<th><strong>Isochrysis sp.</strong></th>
<th><strong>P. lutheri</strong></th>
<th><strong>P. salina</strong></th>
<th><strong>Chlorella sp.</strong></th>
<th><strong>Tetraselmis sp. M8</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>C12</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>0.5</td>
<td>0.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C14</td>
<td>3.5</td>
<td>0.9</td>
<td>0.9</td>
<td>0.4</td>
<td>0.6</td>
<td>10.5</td>
<td>11.6</td>
<td>19.2</td>
<td>11.4</td>
<td>19.4</td>
<td>0.9</td>
<td>4.2</td>
</tr>
<tr>
<td>C15</td>
<td>0.4</td>
<td>0.1</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>C16</td>
<td>33.0</td>
<td>37.3</td>
<td>35.2</td>
<td>22.5</td>
<td>24.7</td>
<td>23.3</td>
<td>26.2</td>
<td>16.4</td>
<td>25.0</td>
<td>24.8</td>
<td>30.9</td>
<td>20.8</td>
</tr>
<tr>
<td>C16:1</td>
<td>26.8</td>
<td>2.5</td>
<td>2.3</td>
<td>1.1</td>
<td>2.9</td>
<td>34.1</td>
<td>29.7</td>
<td>2.0</td>
<td>19.1</td>
<td>3.6</td>
<td>4.4</td>
<td>1.3</td>
</tr>
<tr>
<td>C16:2</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>5.0</td>
<td>2.5</td>
<td>1.5</td>
<td>2.7</td>
<td>0.9</td>
<td>3.1</td>
<td>-</td>
<td>3.4</td>
<td>-</td>
</tr>
<tr>
<td>C16:3</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>2.9</td>
<td>4.0</td>
<td>5.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.8</td>
<td>0.1</td>
<td>-</td>
</tr>
<tr>
<td>C16:4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C17</td>
<td>0.4</td>
<td>0.1</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
<td>1.6</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>2.5</td>
<td>-</td>
</tr>
<tr>
<td>C18</td>
<td>3.0</td>
<td>9.0</td>
<td>8.8</td>
<td>3.0</td>
<td>5.8</td>
<td>5.1</td>
<td>4.5</td>
<td>4.4</td>
<td>4.8</td>
<td>8.3</td>
<td>9.7</td>
<td>10.1</td>
</tr>
<tr>
<td>C18:1</td>
<td>6.0</td>
<td>13.8</td>
<td>15.3</td>
<td>9.1</td>
<td>5.6</td>
<td>5.8</td>
<td>1.7</td>
<td>21.7</td>
<td>1.3</td>
<td>2.0</td>
<td>9.2</td>
<td>13.6</td>
</tr>
<tr>
<td>C18:2</td>
<td>0.9</td>
<td>8.8</td>
<td>19.7</td>
<td>11.7</td>
<td>7.6</td>
<td>0.1</td>
<td>0.2</td>
<td>0.7</td>
<td>-</td>
<td>1.1</td>
<td>7.9</td>
<td>7.0</td>
</tr>
<tr>
<td>C18:3</td>
<td>0.4</td>
<td>15.1</td>
<td>8.8</td>
<td>28.9</td>
<td>33.8</td>
<td>0.0</td>
<td>0.4</td>
<td>3.1</td>
<td>0.1</td>
<td>1.3</td>
<td>22.8</td>
<td>11.1</td>
</tr>
<tr>
<td>C18:4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12.7</td>
</tr>
<tr>
<td>C20</td>
<td>0.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C20:1</td>
<td>-</td>
<td>1.8</td>
<td>2.1</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>5.9</td>
<td>0.1</td>
<td>-</td>
<td>0.8</td>
<td>4.6</td>
<td>-</td>
</tr>
<tr>
<td>C20:4</td>
<td>5.9</td>
<td>2.6</td>
<td>3.3</td>
<td>3.4</td>
<td>-</td>
<td>0.9</td>
<td>1.4</td>
<td>13.9</td>
<td>6.1</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C20:5</td>
<td>18.8</td>
<td>7.2</td>
<td>2.9</td>
<td>10.6</td>
<td>1.2</td>
<td>12.7</td>
<td>14.0</td>
<td>0.0</td>
<td>21.8</td>
<td>16.1</td>
<td>-</td>
<td>10.6</td>
</tr>
<tr>
<td>C22</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22:4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C22:6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>11.8</td>
<td>7.3</td>
<td>10.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total saturated (%)</strong></td>
<td>40.7</td>
<td>47.9</td>
<td>45.6</td>
<td>30.4</td>
<td>31.4</td>
<td>40.5</td>
<td>44.0</td>
<td>39.9</td>
<td>41.1</td>
<td>53.0</td>
<td>43.6</td>
<td>38.9</td>
</tr>
<tr>
<td><strong>Total monounsaturated (%)</strong></td>
<td>32.8</td>
<td>18.2</td>
<td>19.7</td>
<td>10.2</td>
<td>8.6</td>
<td>40.0</td>
<td>31.4</td>
<td>29.6</td>
<td>20.5</td>
<td>5.5</td>
<td>14.4</td>
<td>19.5</td>
</tr>
<tr>
<td><strong>Total polyunsaturated (%)</strong></td>
<td>26.5</td>
<td>34.0</td>
<td>34.7</td>
<td>59.5</td>
<td>60.0</td>
<td>19.5</td>
<td>24.6</td>
<td>30.5</td>
<td>38.3</td>
<td>41.4</td>
<td>42.0</td>
<td>41.7</td>
</tr>
<tr>
<td><strong>Total FAMEs (μg mL⁻¹)</strong></td>
<td>56.1</td>
<td>13.5</td>
<td>13.4</td>
<td>18.7</td>
<td>43.0</td>
<td>29.0</td>
<td>29.5</td>
<td>17.6</td>
<td>17.9</td>
<td>31.4</td>
<td>31.4</td>
<td>57.7</td>
</tr>
<tr>
<td><strong>Total FAME/dry weight (%)</strong></td>
<td>10.6</td>
<td>3.2</td>
<td>10.8</td>
<td>2.5</td>
<td>11.4</td>
<td>-</td>
<td>5.9</td>
<td>3.9</td>
<td>4.0</td>
<td>1.2</td>
<td>5.3</td>
<td>9.9</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0040751.t003
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*
CS-49 and C. calcitrans CS-178 were studied under different conditions to reveal significantly different results. It should be noted that the conditions of the current experimental design were not meant to achieve maximum lipid production but to determine the best lipid producing candidates under standard “unoptimized lab conditions”, which were Nannochloropsis sp. BR2, D. salina and Chlorella sp. BR2. Higher confidence in the data may be obtained by growing cultures completely independently (i.e. experiments carried out separately at different times with a different culture). Subsequent studies may focus on the comparison of best strains under fully optimized and/or large-scale commercial conditions. In our study, Tetraselmis sp M8 was chosen for a scale-up study based on its fast growth rate, culture dominance and ease of harvesting by settling. A comparison of the indoor laboratory conditions to mid-scale (1000 L) outdoor conditions showed that lipid productivity more than doubled under these conditions. Although further long-term studies will be required, these preliminary findings demonstrate the potential for optimization and emphasize that outdoor and large-scale conditions differ strongly from laboratory conditions.

Suitable candidates for biodiesel production require not only high lipid productivity, but also suitable FA content. Recommended FAs for good biodiesel properties 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 (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 mediocre lipid productivity 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 with 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 despite 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) that would allow for a biofinery approach to biodiesel production. It should be noted, however, that microalgal 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 omega-3 FAs and protein-rich biomass. Microalgae possess the potential to produce high amounts of omega-3 FAs and the European Patent Office has documented [31,35,37] that 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. While this may prove useful for biodiesel production, the reduction in PUFA’s is a problem for omega-3 FA production that has been well documented in literature for several other species [34,59,60]. While this may prove useful for biodiesel production, the reduction in PUFA’s is a problem for omega-3 FA production that has been well documented in literature for several other species [34,59,60]. Nevertheless, EPA and DHA contents have been reported to remain consistent despite changes in nutrient level for T. tetrathele [40], which may explain the high levels of PUFA’s observed in Tetraselmis sp.

In a 1000 L-outdoor setting, Tetraselmis sp. M8 was found to have increased µhetero 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)

Acknowledgments
We wish to thank Tania Catalina Atarme-Vega, Kalpesh Sharma, Felicita Vennin, Holger Schulmann, Bart Nijland, Priyanka Nayak, Yamini Kashimshetty, Ekaterina Novak, Miklos Dome and Bernie Degnan for technical assistance and useful discussions. We are also grateful to QSST and CSIRO for provision of additional microalgae strains.

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


Short Communication

Flotation of marine microalgae: Effect of algal hydrophobicity

Sourabh Garga, Yan Li, Liguang Wang, Peer M. Schenk

Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, Brisbane QLD 4072, Australia
School of Chemical Engineering, The University of Queensland, Brisbane QLD 4072, Australia

HIGHLIGHTS

- Algal hydrophobicity has a profound impact on microalgae flotation.
- The ionic strength of flotation medium has little impact on microalgae flotation.
- Algal hydrophobicity can be improved by using a cationic collector.

ARTICLE INFO

Article history:
Received 6 May 2012
Received in revised form 29 June 2012
Accepted 29 June 2012
Available online 16 July 2012

Keywords:
Marine microalgae
Froth flotation
Hydrophobicity
Salinity
Cationic surfactant

ABSTRACT

This study aims to understand the underlying reasons for the poor flotation response of marine microalgae. The flotation performance and hydrophobicity of a freshwater microalga (Chlorella sp. BR2) were compared to those of a marine microalga (Tetraselmis sp. M8) at different salinities in the presence of a cationic collector, tetradecyl trimethylammonium bromide. It was found that microalgal hydrophobicity played a more important role than salinity in determining the flotation performance.

1. Introduction

Microalgae are photosynthetic organisms with great potential to harvest sunlight and convert carbon dioxide into biofuels, health food and animal feed (Chisti 2007; Walker 2005). They have a high photosynthetic efficiency, do not need to compete with edible crops and have comparatively higher oil productivity. Microalgae arguably have become the most promising candidate for the production of biodiesel and other high value products (Chisti 2007; Ota et al. 2009; Schenk et al. 2008). Biofuel production from microalgae can be divided into the following major steps: algae cultivation, biomass harvesting/dewatering, oil extraction and oil conversion to biofuel (Ryan, 2009). The operational costs for dewatering contribute from 20% to 30% to the total biofuel production costs (Brennan and Owende 2010). Dewatering is recognized as a major impediment towards the industrial-scale manufacturing of microalgae bio-products (Danquah et al. 2009; Uduman et al. 2010).

Although the selection of suitable algae harvesting techniques depends largely on the microalgae species and the desired final product, several methods have been proposed for algae harvesting, including centrifugation, filtration, membrane separation process, sedimentation with flocculation, gravity sedimentation, and froth flotation (Phoochinda and White 2003; Uduman et al. 2010). However, most of these methods are of low efficiency and have high capital costs and high energy consumption. For example, centrifugation requires high energy input, a huge cost for large-scale processing which may also damage cells due to high shear forces, resulting in a significant loss of the products of interest (Knuckey et al. 2006). Permeable membranes used for filtration and screening are also easily clogged by tiny microalgae (Uduman et al. 2010) and frequent scraping would significantly shorten the lifetime of these membranes, resulting in high operating costs (Molina Grima et al. 2003). Flocculation seems to be a promising approach for large-scale harvesting, but its application appears to be currently limited to freshwater microalgae. As the ionic strength of water increases, the efficiency of flocculating agent decreases (Uduman et al. 2010). Furthermore, depending on the flocculants, its residues in recycled water may inhibit or prevent renewed algae growth.
Flotation is a proven technology to effectively capture small particles up to 500 μm in aqueous solution using gas bubbles (Matis et al. 1994). It is an effective method to harvest microalgae by taking advantage of their natural characteristics of relatively low density and self-flotation (Phoochinda and White 2003). Also with relatively rapid operation, low space requirements, high flexibility and moderate operational costs, flotation technique has the potential to overcome the bottleneck of feasible microalgal biofuel production (Liu et al. 1999). At present, there are mainly three flotation techniques reported for microalgae harvesting: dispersed air flotation (DAF, bubble diameter 700–1500 μm), dissolved air flotation (DAF, bubble diameter 10–100 μm) and electrolytic flotation (Chisti 2007; Phoochinda et al. 2004; Uduman et al. 2010). Among these techniques, DIAF has been widely used to upgrade coal and minerals at large scale (cell volumes reaching up to 500 m³). DIAF seems to be an economical and efficient technique for harvesting microalgae. At present, algae harvesting by flotation technique has only been developed for freshwater microalgae, such as Chlorella vulgaris and Desmodesmus quadricauda (Chen et al. 1998; Liu et al. 1999; Phoochinda and White 2003; Phoochinda et al. 2004). Although these studies inferred that the flotation efficiency could be affected by salinity (Liu et al. 1999; Phoochinda and White 2003), the flotation of marine microalgae has not been reported yet. In this study, the effectiveness of flotation on marine microalgae harvesting was investigated, through which hydrophobicity as a critical factor and a missing link between flotation performance and algal surface properties was identified.

2. Methods

2.1. Cultivation of algae

Marine microalga Tetraselmis sp. M8 was isolated from the Sunshine Coast, Queensland, Australia (26°39’39”S, 153°6’18”E; Genbank accession number JQ423158) and freshwater microalga Chlorella sp. BR2 was isolated from the Brisbane River, Tennyson, Queensland, Australia (−27°31’21.36”S, 153°0’32.87”E; Genbank accession number JQ423156; Lim et al., 2012). They were cultivated in silica free f/2 medium, under 120 μmol photon m⁻²s⁻¹ with 12-h light/dark cycles, at 26 °C ±1 °C on an orbital shaker (100 rpm). The cultivation was scaled up in two of 14 L cylindrical photobioreactors (one for each) with continuous supply of air and nutrients. When microalgae reached the exponential growth phase, they were nutrient-starved for two days for efficient lipid induction (Hu et al. 2008) and then collected for flotation experiments.

2.2. Dispersed air flotation test

Flotation experiments were carried out using a 1.5-L agitair flotation cell. Air was supplied to the flotation cell through its bottom, where an impeller was placed to provide the agitation necessary for breaking air into bubbles and dispersing them throughout the cell. The bubbles picked up microalgae and rose to the top, forming a microalgae-laden froth, which was subsequently removed manually. Prior to the flotation process, microalgae cultures were stirred vigorously for 2 min. Then each culture was subdivided into aliquots of 1.3 L, weighed and transferred into the flotation cell. The pH of the flotation pulp was adjusted to 9.5 by adding NaOH before adding the collector, tetradecyl trimethylammonium bromide (C14TAB), molecular formula CH₃(CH₂)₁₄N(CH₃)₃(Br−). In the flotation cell, the microalgae suspension was first agitated by stirring at 800 rpm for 5 min. Subsequently, the stirring speed was reduced to 600 rpm and aeration was turned on at a rate of 5 Lmin⁻¹ (superificial air velocity 0.68 cm/s). Four concentrates were sequentially collected at 1, 2, 4, and 6 min. The cell count for each sample was taken in three duplicates by loading 10 μL of sample on a haemocytometer (Brightline, USA), and the average value was reported. The microalgae recovery (Y) and water rejection rate (WRR) were determined using Eqs. (1) and (2).

\[ Y = 1 - \frac{S}{F} \]  
\[ \text{WRR} = \frac{S}{F} \]

where \( S \) is the mass of sink (or tailing left in the flotation cell), \( F \) is the mass of feed, \( s \) is the microalgae concentration in the sink, and \( f \) is the microalgae concentration in the feed.

2.3. Hydrophobicity test

The hydrophobicity of microalgae was measured by using the modified adherence-to-hydrocarbon method (Rosenberg et al. 1980). The test assesses essentially the distribution ratio of cells between water and an organic phase. A total of 4 mL of the algae sample was placed in a test tube to which 1 mL of 98% pure n-hexane was added and shaken vigorously by hand for 1 min; the emulsion was allowed to settle for 2 min. Then, 2 mL were carefully obtained from the bottom aqueous layer of the test tube and its absorbance was read at 620 nm using a spectrophotometer (Hitachi, Model U-2800) to represent the concentration of microalgae. The extractability (H) of the hexane layer on organic substances in the algal suspension was calculated using the following expression:

\[ H = \left( \frac{A_o - A_w}{A_o} \right) \times 100\% \]  

where \( A_o \) is the initial absorbance of the microalgae suspension and \( A_w \) is the absorbance of the aqueous phase after being settled for 2 min.

3. Results and discussion

3.1. Changing collector dosage

The flotation kinetics of freshwater microalga Chlorella sp. (BR2) in freshwater medium and marine microalga Tetraselmis sp. (M8) in seawater medium in the absence of any collector were quite distinct. It was observed that within six minutes, 93% of BR2 could be recovered, whereas only 6% of M8 was recovered. It was hypothesized that M8 had a lower level of natural hydrophobicity than BR2 and that appropriate collectors were needed to render microalgae particles more hydrophobic. Most microalgae are negatively charged at natural pH values (Chen et al., 1998; Phoochinda et al., 2004). Hence, in the present work, a cationic collector, tetradecyl trimethylammonium bromide (C₁₄TAB) was used for subsequent flotation experiments.

At a given flotation time, increasing collector dosage clearly increased the flotation recovery (Fig. 1). The addition of C₁₄TAB increased BR2 recovery to almost 99%, resulting in 30–40% more algae recovery in the first two minutes of flotation (Fig. 1a). A pronounced increase in microalgae recovery was seen when the C₁₄TAB concentration was increased from 1 to 3 ppm. However, there was no further improvement in the recovery when the C₁₄TAB concentration was further increased. The experimental data of cumulative flotation recovery versus flotation time were fitted by using the first-order chemical reaction analogy:

\[ Y = Y_{\text{max}}(1 - e^{-kt}) \]

where \( Y_{\text{max}} \) is the maximum flotation recovery when the flotation time \( t \) approaches infinity, and \( k \) is the flotation rate constant. The
The coefficient of determination ($R^2$) of each curve fit was above 0.965, suggesting that the kinetics of the microalgae flotation process can be satisfactorily modeled by using Eq. (4). The value of $k$ increased considerably with increasing collector dosage from 0 to 3 ppm. Yet further increasing collector dosage to 10 ppm found little change in $k$.

When considering water rejection for BR2, it was observed that within 6 min, 93% water could be removed with only 4.3% of algae cell loss. In other words, the concentration of algae cells could be increased by more than 10 times without significant loss of algae. This confirms that dispersed air flotation is effective for harvesting of freshwater algae.

When the concentration of C14TAB was increased for marine microalga M8, there was a gradual increase in the recovery (Fig. 1b). It increased from 6% to 80% when C14TAB was added from 0 to 80 ppm, suggesting that increasing collector dosage is beneficial for improving marine microalgae flotation recovery. On the other hand, increasing the collector dosage brought about lower water rejection rates. When the C14TAB concentration was at 80 ppm, the water rejection rate was reduced to 60%, associated with overly stable froth.

At a given C14TAB concentration, the flotation recovery of M8 using seawater was much lower than that of BR2 using freshwater. Chen et al. (1998) observed that increasing the ionic strength reduced flotation recovery of microalgae. One could speculate that the relatively large ionic strength of seawater used for M8 flotation may have worsened the bubble-particle attachment. Ducker et al. (1994) found that hydrophobic force is the primary force for attachment of hydrophobic particles to air bubbles and the hydrophobic attraction force could be enhanced by increasing the hydrophobicity of particles. Therefore, a series of studies were performed to elucidate the role of microalgae hydrophobicity in microalgae flotation at various ionic strengths.

### 3.2 Modifying salinity

BR2 was cultivated as usual using freshwater but then 35 ppt NaCl was added and algae were incubated for various times before flotation commenced (Fig. 2a). The control experiment for BR2 flotation was performed without the addition of NaCl and subsequent incubation. The flotation recovery of BR2 in freshwater was 95.1%, and the measured hydrophobicity was 5.3%. When adding 35 ppt NaCl without subsequent incubation, the flotation recovery of BR2 was 97.1% and the measured hydrophobicity was 6.5%. Increasing the incubation time from 0 to 40 min and 22 h (with gentle agitation to avoid settling) had little effect on the flotation recovery and microalgae hydrophobicity. It is clear that ionic strength had little impact on the flotation response of BR2 and its hydrophobicity.

Comparative flotation tests for M8 were performed at three different concentrations of C14TAB in freshwater and seawater (Fig. 2b). M8 was cultivated as usual using seawater. The flotation tests in freshwater were carried out after centrifugation of M8 culture and re-dispersion of the microalgae in freshwater. The difference in flotation recovery of M8 between freshwater and seawater was small (less than 10 percentage points). Likewise, there was little difference between hydrophobicity of M8 in freshwater and seawater. Hence, ionic strength played a rather minor role in determining the flotation of M8 and its hydrophobicity.

It is noteworthy that both flotation recovery and microalgae hydrophobicity increased with increasing collector dosage (Fig. 2b). When seawater was used in flotation, the correlation coefficients between flotation recovery and hydrophobicity were 0.84; when freshwater was used in flotation, the correlation coefficient was 0.91.
coefficient was 0.85. By comparing the hydrophobicity of BR2 and M8 at 3 ppm collector (Fig. 2), one can see that M8 has a lower hydrophobicity (1.3%) than BR2 (5.3%), which corroborates the much poorer flotation performance of marine microalga M8 at 3 ppm C14TAB. The good correlation between microalgae flotation recovery and hydrophobicity suggests that one can increase the flotation performance of microalgae by using more effective collectors which can render microalgal surface more hydrophobic.

4. Conclusions

If no collector was used, the flotation performance of marine microalga M8 was poorer than that of freshwater microalga BR2 because the former had a lower degree of hydrophobicity. The use of cationic surfactant C14TAB enhanced M8’s hydrophobicity and thus its flotation recovery to a satisfactory level but the water rejection rate became low. In contrast, C14TAB was an effective collector for the flotation of BR2 as its flotation recovery and water rejection rate were both high. The ionic strength of flotation medium had little influence on the flotation of M8 and BR2.

Acknowledgements

Financial support for this study, provided by a Linkage Grant (LP0990558) from the Australian Research Council and a CIEF grant by The University of Queensland and Queensland Sea Scallops Trading Pty Ltd, is gratefully acknowledged.

References


Short Communication

Effective harvesting of low surface-hydrophobicity microalgae by froth flotation

Sourabh Garg, Liguang Wang, Peer M. Schenk

Algae Biotechnology Laboratory, School of Agriculture and Food Sciences, The University of Queensland, St Lucia, Queensland 4072, Australia
School of Chemical Engineering, The University of Queensland, St Lucia, Queensland 4072, Australia

Highlights

- Algal hydrophobicity and bubble size are key factors for microalgae flotation.
- Algal hydrophobicity can be improved using cationic surfactants at appropriate pHs.
- A step-wise optimization of algae flotation is demonstrated.

Abstract

Microalgae harvesting by air flotation is a promising technology for large-scale production of biofuel, feed and nutraceuticals from algae. With an adherence-to-hydrocarbon method and two different types of flotation cells (mechanically agitated cell and Jameson cell), microalgal surface hydrophobicity and bubble size were identified to be critical for effective froth flotation of microalgae. Freshwater alga *Chlorella* sp. BR2 showed naturally a high hydrophobicity and an ideal response to flotation. However, many marine microalgae possess a low surface hydrophobicity and are thus difficult to harvest. This paper shows that a step-wise optimization approach can substantially improve the flotation of a low surface hydrophobicity marine microalga, *Tetraselmis* sp. M8, to near full recovery with an enrichment ratio of 11.4.

1. Introduction

Microalgae are considered the most efficient primary producers of biomass. They have great potential to be a future feedstock for producing biofuel and other products as their cultivation does not need to compete for arable land or biodiverse landscapes. Many marine microalgae can use brackish or seawater and are highly efficient producers of lipids. The industrial production of biofuel from microalgae can be divided into three major steps; cultivation, harvesting and processing (Ryan, 2009). Among these, one of the major impediments for commercial-scale production is the downstream processing, where algal biomass has to be concentrated and separated (dewatered) from water for further processing (Christenson and Sims, 2011; Molina Grima et al., 2003). This step can contribute to 20–30% of total biofuel production costs (Molina Grima et al., 2003). Commercial production of microalgal biodiesel requires efficient harvesting and dewatering of algal biomass (Cheng et al., 2010). Various procedures such as flocculation, sedimentation, filtration, flotation, centrifugation and membrane separation have been established for primary dewatering of microalgae from the cultivation medium (Phoochinda and White, 2003).
However, each approach has its own limitation; typically, they are either of low efficiency or high capital cost with excessive energy consumption or cannot be applied at large scale.

Froth flotation presents a promising approach for commercial-scale harvesting of microalgae that compared to other methods, is also relatively low cost (Sharma et al., 2013). It utilizes microalgae’s natural features of relatively low density and self-float (Phoochinda and White, 2003) and is considered a highly versatile method for physically separating particles with a small footprint (Chen et al., 1998; Garg et al., 2012). Microalgal cells are small particles whose size typically ranges from 1 to 20 micron. A missing link between flotation performance and algal surface hydrophobicity has recently been identified and algal hydrophobicity has now been recognized as a major factor determining microalgae flotation efficiency, irrespective of whether these are marine or freshwater microalgae (Garg et al., 2012). Addition of surfactants is commonly used to render algae surface hydrophobic, making it possible to use surfactants as carriers for flotation to separate microalgae from water (Chen et al., 1998; Garg et al., 2012; Uduman et al., 2010).

Froth flotation efficiency can be affected by hydrodynamic and chemical factors. Variables that affect the chemical condition of froth flotation include pH, surfactant type and concentration, as these play important roles affecting the hydrophobicity and electrical charge of particle surfaces (Bulatovic, 2007). Various types of flotation devices which provide different hydrodynamic conditions may also affect flotation separation performance. For example, smaller bubbles generated by using different types of flotation machines can improve fine particle flotation (Yoon, 2000; Zhou et al., 1997).

The Jameson Cell is an advanced flotation apparatus that employs a plunging jet to produce smaller air bubbles than mechanical froth flotation. Triplicate cell counts were carried out for concentrates collected in trays and remaining tailings left in the flotation machine. The Jameson Cell technology was originally applied by Yan and Jameson to treat wastewater (Yan and Jameson, 2004), with microbial removal efficiencies over 98% (on the basis of the difference in concentration between feed and tail).

In the present work, a step-wise comparative study was carried out to understand the effects of different surfactants, pH, cell concentration, and machines on microalgae flotation efficiency with low and high hydrophobicity microalgae. Microalgal recovery for marine microalga Tetraselmis sp. M8 was improved from an initial 6.4% to 97.4% with a satisfactory enrichment ratio of 11.4. Microagal surface hydrophobicity and bubble size were identified as the main underlying causes that improved froth flotation performance.

2. Methods

2.1. Algal culture and characterization

Pure cultures of the green marine microalga Tetraselmis sp. M8 were obtained from a coastal rock pool in Maroochydore, Queensland, Australia (26°39’39”S, 153°6’18”E; Genbank accession number JQ423158) and the green freshwater microalga Chlorella sp. BR2 was isolated from the Brisbane River, Tennyson, Queensland, Australia (27°31’21.36”S, 153°0’32.87”E; Genbank accession number JQ423156) (Lim et al., 2012). Microalgal stocks are maintained in the Algae Biotechnology Laboratory at The University of Queensland, Australia (www.algaebiotech.org). Cultures were grown in silicate free f/2 medium, on an orbital shaker (100 rpm) at 26°C ± 1°C under 120 µmol photon m⁻² s⁻¹ with 12-hour light/dark cycles. Using the same conditions, cultures were scaled up in 20 L polyethylene bags with daily nutrient and continuous air supplies. When microalgal cultures reached the end of the exponential growth phase (less than 20% increase in cell numbers per day), they were nutrient-starved for 2 d for lipid induction (Hu et al., 2008). Subsequently microalgal cultures were used for flotation experiments.

2.2. Froth flotation

Flotation experiments were carried out using a 1.5-L bottom-driven mechanically agitated (Agitair) cell, unless otherwise stated. Microalgal cultures were stirred vigorously for 2 min, before each culture was subdivided into aliquots of 1.3 L, weighed and transferred into the flotation cell. The pH of the flotation pulp was adjusted with HCl or NaOH before adding the collector, tetradeyl trimethyl ammonium bromide (C14TAB) or dodecyl ammonium hydrochloride (DAH). First the microalgal suspension was conditioned by mixing at 800 rpm for 5 min. The agitation rate was 600 rpm or 800 rpm when C14TAB or DAH was used for flotation tests, respectively and the air flow rate was 5 L/min. The mechanical flotation lasted for 6 min.

Once an optimal reagent scheme was determined by the above-described mechanical flotation tests, additional Jameson cell flotation tests were carried out to determine the effect of bubble size (or flotation hydrodynamics) on microalgae flotation. The diameter size of the Jameson Cell used was 150 mm and its orifice diameter was 3.83 mm. A 35-L slurry was fed into the Jameson Cell at a pressure of 150 kPa and an air flow rate of 10 L/min. The Jameson cell flotation time was around 15 min. During this procedure the tailing was continuously recycled to the feed sump and pumped back to the Jameson cell. The Jameson Cell flotation procedure has previously been well described (Bulatovic, 2007; Yan and Jameson, 2004). Microalgal cell count and dry weights were determined for concentrates collected in trays and remaining tailings left in the flotation machine. Triplicate cell counts were carried out for each sample by loading 10 µL of sample on a haemocytometer (Brightline, USA), and the averaged value was determined. Microalgal recovery (Y) was determined using the following equation:

\[ Y = 1 - \frac{W_{\text{tail}}}{W_{\text{feed}}} \]  

where, \( W_{\text{tail}} \) is the wet mass of tailing (or sink), \( W_{\text{feed}} \) is the wet mass of feed, \( Y \) is the microalgal concentration in the tailing, and \( f \) is the microalgal concentration in the feed.

The enrichment ratio (ER) was calculated as the ratio of the concentration of algae in the concentrate to the concentration of algae in the feed.

The following formula was used:

\[ ER = \frac{Y}{1 - W_{\text{WR}}/F} \]  

where \( W_{\text{WR}} \) represents the water rejection rate as equal to \( T/F \).

2.3. Hydrophobicity test

Hydrophobicity (H) of microalgae was quantified by employing a modified adherence-to-hydrocarbon method (Rosenberg et al., 1980). We followed the same procedure as described by Garg et al., 2012 except that the emulsion was allowed to settle for only 20 s.

3. Results and Discussion

An initial comparison of the freshwater microalga Chlorella sp. BR2 with the marine microalga Tetraselmis sp. M8 showed that at pH 9.5, BR2 possessed much higher natural surface hydrophobicity than M8. The flotation recovery of Chlorella sp. BR2 reached more than 90% with a satisfactory enrichment ratio of 13.5, while, interestingly, only 6.4% recovery with an enrichment ratio of only 0.6 was measured for Tetraselmis sp. M8 under identical process conditions. Note that the enrichment ratio of M8 flotation was less than 1, which was most likely caused by the (downward) gravitational sedimentation, which counteracted the (upward) flotation of microalgae.
The baseline flotation response of Tetraselmis sp. M8 in the absence of any modifications was poor, but was considered an ideal model to determine the factors how froth flotation for a low performing microalga can be improved. First, appropriate surfactants can be used as flotation collectors which can render the surface of microalgal cellular structures more hydrophobic. According to published data, most microalgae are negatively charged at a neutral pH, so cationic surfactants are frequently used as flotation collectors for microalgae (Chen et al., 1998; Phoochinda et al., 2004). In the present work, as a first attempt, a range of flotation tests were carried out with C14TAB as a collector. At various concentrations, the influence of C14TAB on algal surface hydrophobicity and flotation performance was systematically analyzed. As shown in Fig. 1, addition of C14TAB considerably increased the cellular surface hydrophobicity and flotation response of Tetraselmis sp. M8. As C14TAB concentration increased from 0 to 80 ppm, algal hydrophobicity increased from 1.2% to 25.0%, the flotation recovery was improved from 6.4% to 81.7%, and the enrichment ratio peaked at 3.4 at a dosage of 50 ppm. While higher C14TAB concentrations considerably improved flotation recovery, it also caused an increased amount of water to be transported to the flotation concentration stream, hence less water was rejected and a lower enrichment ratio was measured. This observation can be explained by the slower liquid drainage in a saline water film confined between air bubbles at higher concentrations of flotation reagents (Wang, 2012). In the present study, the flotation froth became overly stable when increasing C14TAB dosage to high levels. Ideally, a metastable froth is desirable for accomplishing both, a high microalga recovery and a high enrichment ratio.

In seeking for more effective collectors for dewatering of low hydrophobicity marine microalgae, the hydrophobicity of Tetraselmis sp. M8 in aqueous solutions of DAH was first measured. The cultures were also adjusted to different DAH concentrations and pH values. As shown in Fig. 1a, DAH was more capable of increasing M8’s hydrophobicity than C14TAB. Varying DAH dosage from 0 to 50 ppm gradually increased the algal hydrophobicity. Furthermore, at a given DAH concentration, pH 6 resulted in higher hydrophobicity compared to pH 4 and 9.5. The highest algal hydrophobicity (>50%) was measured at 25 ppm DAH at pH 6 or at 50 ppm DAH at pHs 4 – 9.5. Consistent with increased hydrophobicity, higher dosages of DAH also improved the flotation recovery (Fig. 1b). Furthermore, pH had a strong influence on Tetraselmis sp. M8 flotation. At a set DAH concentration, the flotation recovery was always higher at pH 6 than at pH 4 or 9.5. Similar to C14TAB, an increasing amount of DAH collector first augmented the enrichment ratio at 10 ppm before decreasing at higher concentrations (Fig. 1c). The lower enrichment ratio at higher DAH concentrations was caused by the decreased water rejection rate. In particular, at pH 6, when DAH dosage was raised from 10 ppm to 50 ppm, the water rejection ratio was clearly reduced from 93.0% to 68.7%. A pH of 6 resulted in the best overall flotation performance.

However, over the tested DAH concentration range, one could not concurrently measure the highest flotation recovery and the highest enrichment ratio. Hence flotation experimental conditions of 25 ppm DAH and pH 6 were selected as a compromise, where the flotation recovery was 85.0% with an enrichment ratio of 5.6. This assay was then repeated twice under these conditions, and the data were then directly compared with the best flotation performance results for C14TAB experiments that were repeated once. As shown in Fig. 2a, using DAH as a collector resulted in a higher flotation recovery and larger enrichment ratio than C14TAB. It is particularly noteworthy that DAH improved the enrichment ratio to 5.8 with 86.8% algae recovery. By comparison, the enrichment ratio was 3.4 with a flotation recovery of 71.1% when using 50 ppm C14TAB or 2.0 at 80 ppm C14TAB with a flotation recovery of 81.7%.

![Fig. 1. Algal surface hydrophobicity (H), (b) flotation recovery (Y), and (c) enrichment ratio (ER) for harvesting of marine microalga Tetraselmis sp. M8 at different dosages of C14TAB collector at pH 9.5 and DAH at pHs 4, 6 and 9.5. The error bars represent typical sample standard deviation.](image1)

![Fig. 2. (a) Direct comparison of DAH and C14TAB as collector for mechanical flotation performance of Tetraselmis sp. M8; (b) Enrichment ratio versus flotation recovery for microalgal cultures Tetraselmis sp. M8 using mechanical cell and Jameson cell with 25 ppm DAH at pH 6. Lines are drawn to guide the eye, and are expected to follow towards a recovery limitation of 100% at which the enrichment ratio would be 1.](image2)
To determine whether devices considered to be more efficient and capable of generating smaller bubbles may further improve flotation performance, three flotation assays were carried out with a Jameson flotation cell. Conditions included the use of 25 ppm DAH as a collector and a pH-adjusted culture at pH 6 (Fig. 2b). To allow a direct comparison between both flotation cells, data collected under the same chemical conditions are included for the mechanical cell. As shown in Fig. 2b, the Jameson cell resulted in a superior M8 enrichment ratio of 11.4 and a recovery rate of 97.4%. The use of a different flotation machine led to a near doubling of the enrichment ratio and even the flotation recovery increased by at least 10%. For example at a given flotation recovery of 86%, the Jameson cell generated a remarkably higher enrichment ratio (25.9), which was 4.5 times higher than that with the mechanical cell. The desirable flotation performance with the Jameson cell can be attributed to the smaller bubbles (less than 500 micron). The average of bubble size in conventional industrial flotation operations is 1–2 mm. Use of small bubbles can considerably improve fine particle flotation (Yoon, 2000; Zhou et al., 1997). Smaller bubbles can interact with mineral particles at higher collision probability, higher attachment probability and lower detachment probability, lower ascending rate and higher free surface energy, which are all desirable for flotation. However, other properties of the Jameson cell may also contribute. For example, the Jameson cell has intense mixing with small bubbles, tailings recycling, no mechanical agitation and no external air supply. Further research should be conducted to better understand the flotation performance of marine microalgae using the Jameson Cell technology and whether this can be applied at large industrial scales. To determine dependency of flotation recovery and algal surface hydrophobicity, both parameters were plotted in Fig. 3. It shows that when C14TAB or DAH was used as the collector, a strong correlation exists between flotation recovery and algal hydrophobicity. Although C14TAB could be used to improve the hydrophobicity of the marine microalgae Tetraselmis sp., DAH was much more effective for rendering the surface of this microalgal hydrophobic. Typically, a higher packing density of the collector hydrocarbon tails on the particle surface should result in a stronger surface hydrophobicity. A collector that can be better adsorbed onto microalgal cellular surfaces may also enable the formation of microalgal aggregate, which is favorable for improving fine particle flotation. Xu and Yoon, (1990) showed that hydrophobic coagulation, which is driven by the hydrophobic force, also plays an important role in flotation, and the kinetics of coagulation increase with increasing particle hydrophobicity.

The observed correlation between microalgae flotation recovery and hydrophobicity suggests that considerable increases in flotation performance of microalgae can be expected by using more effective collectors that can increase the hydrophobicity of microalgal surfaces. Quantification of hydrophobicity for microalgae presents a simple and effective assay that allows screening of flotation collectors and other chemical conditions of microalgal cultures, prior to using elaborate froth flotation assays. Results from the present study demonstrate that even low hydrophobicity marine microalgae can be harvested by froth flotation. Culture properties, chemical reagents and bubble size (hydrodynamics) are important for the flotation of marine microalgae. Further studies need to determine whether collectors can possibly interfere with downstream processing (including water recycling) and whether application of froth flotation for microalgae harvesting can help establish large-scale commercial cultivation.

4. Conclusions

Microalgae with low surface hydrophobicity are difficult to harvest by flotation separation. C14TAB as collector improved the flotation recovery of marine microalgae, Tetraselmis sp. M8 in a mechanically-agitated cell from 6.4% to 81.7%, but with an unsatisfactory enrichment ratio of 2.0. DAH rendered cells more hydrophobic, improving the enrichment ratio to 5.8 with 86.8% recovery. A Jameson cell with relatively small bubbles resulted in an enrichment ratio of 11.4 with 97.4% recovery. It is concluded that surface hydrophobicity and bubble size are key factors affecting algae flotation, and a step-wise optimization can lead to effective flotation separation of difficult-to-harvest microalgae.

Acknowledgements

Financial support for this study, provided by the Australian Research Council and a CIEF grant by The University of Queensland, is gratefully acknowledged.

References