

## Video Article

# Experimental Protocol for Biodiesel Production with Isolation of Alkenones as Coproducts from Commercial *Isochrysis* Algal Biomass

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## Abstract

The need to replace petroleum fuels with alternatives from renewable and more environmentally sustainable sources is of growing importance. Biomass-derived biofuels have gained considerable attention in this regard, however first generation biofuels from edible crops like corn ethanol or soybean biodiesel have generally fallen out of favor. There is thus great interest in the development of methods for the production of liquid fuels from domestic and superior non-edible sources. Here we describe a detailed procedure for the production of a purified biodiesel from the marine microalgae *Isochrysis*. Additionally, a unique suite of lipids known as polyunsaturated long-chain alkenones are isolated in parallel as potentially valuable coproducts to offset the cost of biodiesel production. Multi-kilogram quantities of *Isochrysis* are purchased from two commercial sources, one as a wet paste (80% water) that is first dried prior to processing, and the other a dry milled powder (95% dry). Lipids are extracted with hexanes in a Soxhlet apparatus to produce an algal oil ("hexane algal oil") containing both traditional fats (*i.e.*, triglycerides, 46-60% w/w) and alkenones (16-25% w/w). Saponification of the triglycerides in the algal oil allows for separation of the resulting free fatty acids (FFAs) from alkenone-containing neutral lipids. FFAs are then converted to biodiesel (*i.e.*, fatty acid methyl esters, FAMES) by acid-catalyzed esterification while alkenones are isolated and purified from the neutral lipids by crystallization. We demonstrate that biodiesel from both commercial *Isochrysis* biomasses have similar but not identical FAME profiles, characterized by elevated polyunsaturated fatty acid contents (approximately 40% w/w). Yields of biodiesel were consistently higher when starting from the *Isochrysis* wet paste (12% w/w vs. 7% w/w), which can be traced to lower amounts of hexane algal oil obtained from the powdered *Isochrysis* product.

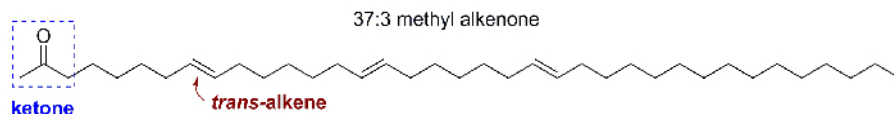
## Video Link

The video component of this article can be found at <http://www.jove.com/video/54189/>

## Introduction

There has recently been a great resurgence of interest in biofuels from algae, particularly for the production of liquid fuels such as biodiesel<sup>1</sup> and other biomass-derived oils.<sup>2</sup> Proposed benefits include the avoidance of certain food vs. fuel controversies<sup>3</sup> and reportedly higher productivities and CO<sub>2</sub> mitigation capabilities than traditional agricultural crops.<sup>4</sup> This follows the nearly 20 year United States Department of Energy's Aquatic Species Program (ASP) started in 1978 for the purpose of investigating transportation fuel from algae. As outlined in Sheehan's report,<sup>5</sup> the program ended in 1996 primarily because projected costs were not competitive with crude petroleum at that time (\$18.46 per barrel (159 L)). While the cost of petroleum has increased dramatically since then (\$87.39 per barrel in 2014)<sup>6</sup>, which is connected to the renaissance in algal biofuel research, some have argued that nonetheless algal biofuels will prove too costly.<sup>7</sup> As one strategy to offset biofuel production costs, the notion of value-added coproducts has emerged among both critics<sup>7,8</sup> and proponents<sup>9,10</sup> and features as one of the key reasons for pursuing algal biofuels in the United States Department of Energy (DOE) "National Algal Biofuels Technology Roadmap".<sup>11</sup>

Here we describe a method for the coproduction of two separate fuel streams from commercial *Isochrysis* microalgae. We have focused on *Isochrysis* in part because it is already produced industrially, harvested for purposes of mariculture, and also because *Isochrysis* is one of only a few species of algae that in addition to traditional lipids (*i.e.*, fatty acids) biosynthesize a unique class of compounds known as polyunsaturated long-chain alkenones.<sup>12</sup> Alkenone structures are characterized by very long hydrocarbon chains (36-40 carbons), two to four non-methylene interrupted *trans*-double bonds, and a methyl or ethyl ketone (**Figure 1**). Alkenone unsaturation is sensitive to the algae growing temperature,<sup>13,14</sup> such that the proportion of the diunsaturated C37 methyl alkenone (the so-called "unsaturation index") can be used as a proxy for past sea surface temperatures.<sup>15-20</sup> Alkenones are thought to reside in cytoplasmic lipid bodies and can be more abundant than triglycerides (TAGs).<sup>21,22</sup> Under nitrogen or phosphorous limitation, up to 10-20% of cell carbon in the stationary phase is accumulated as alkenones.<sup>23,24</sup> From an evolutionary standpoint, alkenones may have been favored over TAGs because their *trans*-double bond geometry provides a more stable form of energy storage.<sup>21</sup>



**Figure 1. Structures of polyunsaturated long-chain alkenones.** Common alkenone methyl 37:3 isolated from *Isochrysis* exemplifying long hydrocarbon chain lengths (36 - 40 carbons), *trans* non-methylene interrupted double bonds, and terminating in a methyl or ethyl ketone. Nomenclature is similar to fatty acids where #:# refers to number of carbons:number of double bonds. [Please click here to view a larger version of this figure.](#)

We argue that alkenones represent a promising renewable carbon feedstock from a common algae with a history of industrial cultivation.<sup>25</sup> Biodiesel produced directly from the total lipid extract of *Isochrysis* contains a significant amount (10-15% w/w) of alkenones and contamination by these high-melting compounds results in poor cold flow fuel properties. However, using the saponification/extraction techniques described here, alkenones can be removed and recovered thereby improving the biodiesel quality while producing a secondary product stream. Recently we demonstrated the conversion of alkenones to a liquid fuel by cross-metathesis with 2-butene (butenolysis).<sup>26</sup> The butenolysis reaction employs a commercial ruthenium metathesis-initiator, occurs rapidly at low temperature, and cleanly delivers a predictable mixture of jet fuel range hydrocarbons. This reaction is performed in parallel with biodiesel synthesis from fatty acids, representing the first steps toward a "biorefinery" approach<sup>27</sup> for commercially viable *Isochrysis* biofuel production.

## Protocol

### 1. Microalgae and Biomass Preparation

Note: The marine microalgae *Isochrysis* sp. "T-iso" used in the present study can be purchased (see List of Materials). Multi-kilogram quantities of *Isochrysis* can be purchased as a frozen wet paste (*Iso*-paste) containing approximately 80% water and 20% biomass, and is dark green/near black in color with a pungent odor smelling of the sea. *Isochrysis* can also be purchased as a dry (95% dry) yellow-brown powder (*Iso*-powder) with a similar odor.

1. In order to dry the *Isochrysis* paste, open a 1 kg package by cutting a 1 - 2 inch hole in the corner of the plastic packaging using scissors.
2. Squeeze approximately 300 g of this *Isochrysis* paste through the hole into a 150 mm x 75 mm crystallizing dish to create a thin layer (~20 mm).
3. Leave the paste to air-dry at room temperature until it becomes dry and flaky (typically, 48 - 96 hr).  
Note: Actual drying times may vary and are dependent on temperature. However, no difference was noticed in the yields or product quality with even longer drying times (up to two weeks). The drying process may also be made more uniform and/or accelerated by placing the crystallizing dish on a warm plate (30 - 40 °C).
4. Scrape the dry biomass from the crystallizing dish using a spatula and collect into a cellulose extraction thimble (Length: 123 mm, 43 mm ID). Record the weight of the dry *Isochrysis* biomass.

### 2. Soxhlet Extraction of Dry *Isochrysis* Biomass

1. Load an *Isochrysis*-containing cellulose extraction thimble (typically 50 - 60 g dry biomass) into a Soxhlet extraction apparatus.
2. Fill the Soxhlet flask with hexanes (400 ml), turn on the condenser water and heat source, and allow the Soxhlet to cycle for 24 - 48 hr (until the color of the solvent has gone from dark green to a faint yellow).
3. Turn off the heat and allow the apparatus to cool to room temperature, then disconnect the flask from the Soxhlet extractor.
4. Remove the hexanes using a rotary evaporator and record the weight of the hexanes-extractable material ("hexane algal oil" (h-AO)).

### 3. Saponification of the Algal Oil and Separation of Fatty Acids and Neutral Lipids

1. Redissolve the h-AO in the same round bottom flask from Step 2.4 above with methanol:dichloromethane (2:1, volume = 10 x mass of algal oil).
2. Add a stir bar and attach a reflux condenser (Coil: 500 mm length).
3. Add H<sub>2</sub>O (volume = 2.67 x mass of algal oil) and KOH (50% w/w algal oil) and heat the contents with stirring to 60 °C for 3 hr.
4. After cooling to room temperature, remove the organic solvents (methanol and dichloromethane) on a rotary evaporator.
5. Transfer the remaining aqueous mixture by pouring into a 1-L separatory funnel. Add hexanes (equivalent in volume to the aqueous solution), shake the separatory funnel, and allow the layers to separate.
6. Drain the lower aqueous layer into an Erlenmeyer flask, and pour off the top organic phase into a separate Erlenmeyer.
7. Repeat steps 3.5 and 3.6 until the organic layer is colorless (typically 1-2 more times).
8. Concentrate the combined organic extracts on a rotary evaporator to isolate the neutral lipids as a greenish solid (mp. ≈ 60 - 70 °C).
9. Acidify the aqueous phase with HCl (6 M, until pH ~ 2 as indicated by pH paper).
10. Extract the free fatty acids (FFAs) from the acidified aqueous phase with hexanes (equivolume to the aqueous phase) using a 1 L separatory funnel as described in steps 3.5 and 3.6.
11. Remove the hexanes on a rotary evaporator to obtain the FFAs as a dark green near-black oily residue (liquid at temperatures >30 °C).

### 4. Acid-catalyzed Esterification of Free Fatty Acids and Production of a Green Biodiesel

1. Transfer the FFAs using methanol:chloroform (1:1, 6 x volume of algal oil) to first dissolve the FFAs and then by pouring into a thick-walled high pressure reaction flask equipped with a stir bar.

2. Add concentrated H<sub>2</sub>SO<sub>4</sub> (20% w/w algal oil), seal the flask, and heat the mixture to 90 °C while stirring for 1 hr.
3. After cooling to room temperature, transfer the mixture by pouring into a separatory funnel.
4. Add H<sub>2</sub>O (2 x volume of algal oil), shake the separatory funnel, and allow the phases to separate.
5. Drain the bottom layer into a pre-weighed round bottom flask and concentrate on a rotary evaporator. Record the mass of the resulting biodiesel.
6. Analyze the fatty acid profile by gas chromatography with flame-ionization detector (GC-FID)<sup>28</sup> (gas chromatograph equipped with a DB-88 [(88% cyanopropyl) methylarylpolysiloxane] column (30 m x 0.25 mm ID x 0.20 μm film thickness).  
Note: Common fatty acid methyl esters are verified by retention time comparison with authentic samples obtained commercially. Additionally, gas chromatography-mass spectrometry (GC-MS; gas chromatograph coupled to a mass selective detector) is performed under identical conditions of temperature program and column to analyze components such as C18:4 for which no authentic standards are available with the results applied to GC quantitation.

## 5. Biodiesel Decolorization

1. Heat the dark green colored biodiesel to 60 °C in a round bottom flask equipped with a stir bar.
2. Add montmorillonite K 10 (MK10) powder (10-20% w/w of the biodiesel) and stir for 1 hr.
3. Remove the round bottom from the heat and allow the solution to cool to room temperature.
4. Prepare a filtration apparatus consisting of a round bottom flask and filter funnel containing a cellulose filter paper (Ash 0.007%).
5. Pour the cooled decolorized biodiesel through the filter funnel using a minimal amount of hexanes to rinse the round bottom flask.
6. Disconnect the filter funnel from the round bottom flask (this now contains a hexanes solution of the decolorized biodiesel) and remove the hexanes with a rotary evaporator to afford an orange/red biodiesel.
7. Store samples at 4 °C during which time some settling of insoluble material (~10% w/w) will occur.
8. Remove the insoluble material by decanting or filtration as described in step 5.4 and 5.5 to produce a clear homogeneous biodiesel for analysis.

## 6. Isolation and Purification of Alkenones from the Neutral Lipids

1. Dissolve the neutral lipids (from Step 3.8) in a minimal amount of dichloromethane (approximately 50 ml for 10 g neutral lipids) and add the solution with a pipet to the top of a chromatography column (O.D 60 mm, ID 55 mm, length 18") containing silica gel (230-400 mesh, 100 g).
2. Elute the solution through the silica with pressure (~5 psi) using dichloromethane (approximately 150 ml) as solvent and collect the eluent in a 250 ml round bottom flask.
3. Remove the dichloromethane with a rotary evaporator to give an orange-colored solid.
4. Recrystallize the solid using hexanes by adding approximately 100 ml of boiling hexanes followed by incremental additional amounts of boiling hexanes until the solution is homogeneous (total volume ~ 150 ml). Then, slowly cool the solution to room temperature to promote crystallization.
5. Collect the crystallized alkenones using a filtration apparatus as described in step 5.4 using a small amount of cold (0 °C) hexanes to rinse the flask.

## Representative Results

Prior to processing, the *Isochrysis* paste (*Iso*-paste) was first dried. This was conveniently accomplished on larger scale by adding the *Iso*-paste to a large crystallizing dish and allowing the material to air-dry at room temperature. During drying, some pooled water forms (generally reddish colored) that can be removed by decanting or pipetting to accelerate the drying process. After approximately 48 - 96 hr, the now dry *Isochrysis* could be scraped out of the crystallizing dish and obtained as a black/green flaky material with a seaweed-like smell (**Figure 2**). Yields of dry biomass were generally 20% w/w of the paste as advertised. By contrast, the powdered *Isochrysis* product (*Iso*-powder) was a yellow-brown, finely milled, dry powder (95% dry) that was used directly without further processing (**Figure 2**).



**Figure 2. Comparison of commercial *Isochrysis*.** *Isochrysis* paste (80% wet) is spread along the bottom of a crystallizing dish and left to air-dry at room temperature for 48-96 hr before processing. The resulting dried *Isochrysis* is obtained as a dark-colored flaky material (right) that is different in appearance than the commercial dry powder *Isochrysis* (left). [Please click here to view a larger version of this figure.](#)

Extraction of either the dried *Iso*-paste or *Iso*-powder by Soxhlet with hexanes gave, after removal of the hexanes, algal oils (h-AO) that were similar in appearance as dark-green/near-black solids (mp. ~ 50-60 °C). Yields of h-AO when starting from the paste ("*Iso*-paste-hAO") were typically 20% w/w of the dry *Isochrysis* biomass, consistent with our previous results,<sup>26</sup> whereas yields of h-AO by Soxhlet extraction of the commercial powdered *Isochrysis* ("*Iso*-powder-hAO") were 15% w/w (**Table 1**).

Product (g)	<i>Iso</i> -paste	<i>Iso</i> -powder-1	<i>Iso</i> -powder-2
Dry Biomass	30	20	20
Hexane Algal Oil	5.86	2.87	3.11
FFAs	3.52	1.34	1.38
Neutral lipids	2.34	1.38	1.61
Alkenones	0.94	0.63	0.74

Notes: *Iso*-powder-1 and *Iso*-powder-2 represent results from two samples of powdered *Isochrysis* that were processed in parallel. For other reports with yields of these products from *Iso*-paste see references 26, 32, and 33.

**Table 1. Product yields from commercial *Isochrysis* biomass.**

Acylglycerols in the h-AO were converted to the corresponding water-soluble carboxylate salts (*i.e.*, soaps) upon addition of aqueous KOH in methanol/CH<sub>2</sub>Cl<sub>2</sub>. Neutral lipids including alkenones were then extracted from this aqueous mixture by selective partitioning with hexanes. After removal of the neutral lipids, reacidification of the soaps then produced the corresponding free fatty acids (FFAs) that could be similarly extracted from the aqueous phase with hexanes. The overall mass recoveries for combined FFAs and neutral lipids from either *Iso*-paste-hAO or *Iso*-powder-hAO were consistently near quantitative. However, the ratio of products (*i.e.*, neutral lipids + FFAs) was different. From the *Iso*-paste-hAO we obtained 60% (w/w) FFAs and 40% (w/w) neutral lipids (**Table 1**). Conversely, *Iso*-powder-hAO proved enriched in neutral lipids (average = 54% neutral lipids + 46% FFAs) as detailed in **Table 1**.

Esterification of the FFAs with H<sub>2</sub>SO<sub>4</sub> and methanol then produced fatty acid methyl esters (FAMES, *i.e.*, biodiesel) as a dark green near black oily liquid in greater than 90% yield (**Figure 3**). Decolorization by heating over Montmorillonite K10<sup>29</sup> (MK10) clay then gave a yellow/orange product, similar in appearance to other commercial biodiesel fuels (see List of Materials) (**Figure 3**). Results from the FAME analysis of decolorized *Isochrysis* biodiesel fuels are shown in **Table 2**.



**Figure 3. Comparison of *Isochrysis* and soybean biodiesel fuels.** Green *Isochrysis* biodiesel (middle) is produced by esterification of extracted and purified free fatty acids. Decolorization produces a product (right) with similar properties to commercial biodiesel (left). [Please click here to view a larger version of this figure.](#)



FAME <sup>A</sup>	Iso-paste	Iso-powder
14:00	16.4	19.4
14:01	-	0.3
15:00	trace	0.3
16:00	10.1	8.8
16:1 Δ9	7.6	5.5
16:02	ND	0.3
16:03	ND	0.5
18:00	Trace	0.2
18:1 <sup>B</sup>	12.1	14.3
18:02	8.1	7.1
18:3 <sup>C</sup>	8.5	13.5
18:04	19.8	10.4
18:05	ND	3
20:05	ND	-
22:05	ND	2
22:06	6.9	11
Σ <sup>D</sup>	89.8	96.2

Notes: <sup>A</sup>Fatty acid nomenclature is #carbons:#cis-double bonds. <sup>B</sup>Combined 18:1 Δ9 + 18:1 Δ11. <sup>C</sup>Combined Δ6,9,12 and Δ9,12,15 isomers. <sup>D</sup>The remaining material is roughly 50:50 other FAMEs and non-FAME components (Total ~95% FAME). ND = Not detected.

**Table 2. FAME composition of biodiesel produced from commercial Iso-paste and Iso-powder Isochrysis biomass.**

Neutral lipids were obtained as a greenish solid mixture at 40% w/w from the Iso-paste-hAO and 54% (avg.) from the Iso-powder-hAO (Table 1). Filtering the dissolved neutral lipids through silica using DCM gave after removal of the solvent, a reddish/orange solid that could be recrystallized with hexanes to afford analytically pure alkenones as a white solid. This procedure resulted in 16% (w/w) isolated yield of alkenones from Iso-paste-hAO and 25% yield from Iso-powder-hAO (Table 1).

## Discussion

*Isochrysis* is one of only a select number of algal species farmed industrially, harvested as a primary component of shellfish feed, and therefore representative of the scale necessary for biofuel production. The availability of the algae utilized and standard methods employed in this study, make the protocol presented widely accessible to other groups for further investigations. Critical steps include air-drying the algae (as opposed to lyophilizing<sup>33</sup>), solvent extractions, saponification, and esterification. Through these operations one can examine yields of lipids and other coproducts from the various *Isochrysis* available<sup>30</sup>. It is anticipated that these may differ as a result of differing strains and cultivation methods,<sup>31</sup> and may also be impacted by the nature of the product and any additional processing (e.g., drying or freezing) utilized by the supplier. As we demonstrate here, the protocol developed can be successfully applied to different types of *Isochrysis* products, ranging from a wet paste to a dry milled powder. Yields of biodiesel were however lower from the powdered biomass (7% w/w dry biomass vs. 12% w/w from the dried paste), which corresponds with lower amounts of algal oil (h-AO) extracted. This may suggest that an alternative extraction protocol<sup>32</sup> other than a Soxhlet apparatus may be better suited for dry powdered *Isochrysis* products. The *Isochrysis* powder used in this study is advertised as containing 23-25% lipids, which similar to what we have experimentally obtained from dried *Isochrysis* paste.<sup>33,34,26</sup>

Despite the different colors of the starting dry biomass, the Iso-paste-hAO and Iso-powder-hAO were essentially indistinguishable, both dark green/near black solids with melting points of approximately 50 °C. Interestingly, the ratio of FFAs to neutral lipids within the two hexane extracts was different. After saponification and separation of the neutral lipids, we obtained 60% (w/w) FFAs and 40% (w/w) neutral lipids from the Iso-paste-hAO. The Iso-powder-hAO produced on average 46% (w/w) FFAs and 54% (w/w) neutral lipids. The results suggest that either the starting powdered biomass may contain higher amounts of neutral lipids relative to FA derivatives than the *Isochrysis* paste, or that Soxhlet extraction of powdered *Isochrysis* is somewhat selective for neutral lipids.

Not only were the yields of products obtained from the two commercial *Isochrysis* biomasses different, but also the fatty acid profiles of the resulting biodiesel. This is important, as the fuel properties of a biodiesel are directly dependent on the nature and contents of individual FAMEs.<sup>35</sup> To be commercialized, all biodiesel must conform to the standards described in the documents ASTM D6751 or EN 14214 in the U.S or Europe respectively. Specifications include ranges for lubricity and kinematic viscosity, and minimum values for cetane number and oxidative stability. Other important recommendations are related to cold flow properties in the form of a cloud point (CP) or cold filter plugging point (CFPP). We have previously reported results from the comprehensive fuel testing of biodiesel prepared from Iso-paste.<sup>36</sup> Since the FAME profile of biodiesel produced from the Iso-powder in this study is similar to those previously tested, we can predict certain fuel properties to be similar for both biodiesel fuels. For instance, polyunsaturated fatty acids (PuFAMEs, more than two double bonds) account for approximately 40% of both FAME mixtures (35.2% and 39.9%, Table 2). This will result in poor oxidative stability and favorable cold-flow.<sup>35</sup> There are, however,

slight differences in the FAME profiles of the two biodiesel fuels. Biodiesel produced from the powdered *Isochrysis* contained higher amounts of 14:0 (19.4 mg/g vs. 16.4 mg/g), 18:3 (13.5 mg/g vs. 8.5 mg/g), and 22:6 (11.0 mg/g vs. 6.9 mg/g) FAMES, yet lower amounts of 18:4 (10.4 mg/g vs. 19.8 mg/g). The extent of the impact of these differences on the various fuel properties contained in the ASTM standards remains to be investigated.

The initial biodiesel obtained from both commercial *Isochrysis* algae were similarly dark green in color which can be explained by the presence of chlorophylls.<sup>36</sup> Chlorophyll and its derivatives have been reported to have a negative effect on the stability of vegetable oils and their corresponding biodiesel fuels.<sup>36,29</sup> Based on the method of Issariyakul and Dalai for decolorizing greenseed canola oil in connection with biodiesel production,<sup>29</sup> stirring our green biodiesel over 10% (w/w) MK10 at 60 °C for 1 hr resulted in a dramatic reduction in pigment content by visual inspection (ref. **Figure 2**). Mass recoveries from the decolorization process were on average 90%.

Yields of purified alkenones from *Iso*-paste-hAO and *Iso*-powder-hAO neutral lipids were comparable at 40% and 46% w/w respectively (**Table 1**). Since neutral lipids represent a higher proportion of material contained in the *Iso*-powder-hAO (54% w/w vs. 40% w/w), alkenone yield from the *Iso*-powder-hAO exceeds the *Iso*-paste-hAO alkenone yield by approximately 10% (25% w/w vs. 16% w/w). However, considering that yields of the *Iso*-powder-hAO itself were lower than *Iso*-paste-hAO (15% vs. 20% w/w), overall yields of alkenones from both dry *Isochrysis* biomasses are more similar ( $0.2 \times 0.4 \times 0.4 = 3.2\%$  w/w from dried *Isochrysis* paste and  $0.15 \times 0.54 \times 0.46 = 3.7\%$  from *Isochrysis* powder).

## Disclosures

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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## References

- Ahmad, A.L., Mat Yasin, N.H., Derek, C.J.C., Lim, J.K. Microalgae as a sustainable energy source for biodiesel production: A review. *Renew. Sustain. Energy Rev.* **15**(1), 584-593 (2011).
- Vardona, D. R., Sharmab, B.K., Blazinaa, G.,V., Rajagopalanb, K., Strathmann, T.J. Thermochemical conversion of raw and defatted algal biomass via hydrothermal liquefaction and slow pyrolysis. *Bioresour. Technol.* **109**, 178-187 (2012).
- Tenenbaum, D.J. Food vs fuel: Diversion of crops could cause more hunger. *Environ. Health Perspect.* **116**, A254-A257, PMID: PMC2430252 (2008).
- Pienkos, D.T., Darzins, A. The promise and challenges of microalgal-derived biofuels. *Biofuels, Bioprod. Biorefin.* **3**(4), 431-440 (2009).
- First Crude Oil Purchase Price*. U.S Energy Information Administration. [http://www.eia.gov/dnav/pet/hist/LeafHandler.ashx?n=PET&s=F000000\\_\\_3&f=A](http://www.eia.gov/dnav/pet/hist/LeafHandler.ashx?n=PET&s=F000000__3&f=A). accessed September 30, 2015, (2015).
- Sheehan, J., Dunahay, T., Sheehan, J., Dunahay, T., Benemann, J., Roessler, P.A. *A look back at the U.S. Department of Energy's Aquatic Species Program: Biodiesel from algae*. National Renewable Energy Laboratory, report NREL/TP-580-24190 (1996).
- Petkov, G., Ivanova, A., Iliev, I., Vaseva, I. A critical look at the microalgae biodiesel. *Eur. J. Lipid Sci.* **114**(2), 103-111 (2012).
- Beilen, J.B. Why microalgal biofuels won't save the internal combustion engine. *Biofuels, Bioprod. Biorefin.* **4**, 41-52 (2010).
- Chisti, Y. Biodiesel from microalgae. *Biotechnol. Adv.* **25**(3), 294-306, (2007).
- Wijffles, R.H., Barbosa, M.J. An outlook on microalgal biofuels. *Science.* **329**, 796-799 (2010).
- Ferrell, J. Sarisky-Reed, V. *National Algal Biofuels Technology Roadmap*. (Eds. D. Fishman, R. Majumdar, J. Morello, R. Pate, J. Yang), United States Department of Energy, [http://www1.eere.energy.gov/bioenergy/pdfs/algal\\_biofuels\\_roadmap.pdf](http://www1.eere.energy.gov/bioenergy/pdfs/algal_biofuels_roadmap.pdf) (accessed August 24, 2015) (2010).
- Patterson, G.W., Tsitsa-Tsardis, E., Wikfors, G.H., Gladu, P.K., Chitwood, D.J., Harrison, D. Sterols and alkenones of *Isochrysis*. *Phytochem.* **35**(5), 1233-1236 (1994).
- Volkman, J.K., Eglinton, G., Corner, E.D.S. Long-chain alkenes and alkenones in the marine coccolithophorid *Emiliania huxleyi*. *Phytochem.* **19**, 2619-2622 (1980).
- Conte, M.H., Thompson, A., Lesley, D., Harris, R.P. Genetic and physiological influences on the alkenone/alkenoate versus growth temperature relationship in *Emiliania huxleyi*. and *Gephyrocapsa Oceanica*. *Geochim. Cosmochim. Acta.* **62**(1), 51-68 (1998).
- Brassell, S.C., Eglinton, G., Marlowe, I.T., Pflaumann, U., Sarnthein, M. Molecular stratigraphy: a new tool for climatic assessment. *Nature.* **320**, 129-133 (1986).
- Marlowe, I.T., Brassell, S.C., Eglinton, G., Green, J.C. Long chain unsaturated ketones and esters in living algae and marine sediments. *Org. Geochem.* **6**, 135-141 (1984).
- Prahl, F.G., Wakeham, S.G. Calibration of unsaturation patterns in long-chain ketone compositions for palaeotemperature assessment. *Nature.* **330**, 367-369 (1987).
- Eglinton, G., Bradshaw, S.A., Rosell, A., Sarnthein, M., Pflaumann, U., Tiedemann, R. Molecular record of secular sea surface temperature changes on 100-year timescales for glacial terminations I, II and IV. *Nature.* **356**, 423-426 (1992).
- Kirst, G., Ruhland, G., von Storch, I., Rosell-Melé, A. Calibration of the alkenone paleotemperature index U37K' based on core-tops from the eastern South Atlantic and the global ocean (60°N-60°S). *Geochim. Cosmochim. Acta.* **62**(10), 1757 - 1772 (1998).
- Volkman, J.K., Barrerr, S.M., Blackburn, S.I., Sikes, E.L. Alkenones in *Gephyrocapsa oceanica*: Implications for studies of paleoclimate. *Geochim. Cosmochim. Acta.* **59**(3), 513-520 (1995).

21. Eltgroth, M.L., Watwood, R.L., Wolfe, G.V. Production and cellular localization of neutral long-chain lipids in the haptophyte algae *Isochrysis Galbana* and *Emiliania Huxleyi*. *J. Phycol.* **41**, 1000-1009 (2005).
22. Volkman, J.K., Everitt, D.A., Allen, D.I. Some analyses of lipid classes in marine organisms, sediments and seawater using thin-layer chromatography-flame ionisation detection. *J. Chromatogr. A.* **356**, 147-162 (1986).
23. Epstein, B. L.; D'Hondt, S.; Quinn, J. G.; Zhang, J.; Hargraves, P. An effect of dissolved nutrient concentrations on alkenone-based temperature estimates. *Paleoceanography.* **13**(2), 122-126 (1998).
24. PrahI, F.G., Sparrow, M.A., Wolfe, G.V. Physiological impacts on alkenone paleothermometry. *Paleoceanography.* **18**(2), 1025-1031 (2003).
25. Sachs, D., Sachs, J.P. Inverse relationship between D/H fractionation in cyanobacterial lipids and salinity in Christmas Island saline ponds. *Geochim. Cosmochim. Acta.* **72**(25), 793-806 (2008).
26. Neil, G.W., Culler, A.R., Williams, J.R., Burlow, N.P., Gilbert, G.J., Carmichael, C.A., Nelson, R.K., Swarthout, R.F., Reddy, C.M. Production of jet fuel range hydrocarbons as a coproduct of algal biodiesel by butenolysis of long-chain alkenones. *Energy Fuels.* **29**(2), 922-930 (2015).
27. Foley, P.M., Beach, E.S., Zimmerman, J.B. Algae as a source of renewable chemicals: opportunities and challenges. *Green Chem.* **13**, 1399-1405 (2011).
28. Razon, L.F., Bacani, FT., Evangelista, R.L., Knothe, G. Fatty acid profile of kenaf seed oil. *J. Am. Oil Chem. Soc.* **90**(6), 835-840 (2013).
29. Issariyakul, T., Dalai, A.K. Biodiesel production from greenseed canola oil. *Energy Fuels.* **24**, 4652-4658 (2010).
30. Nalder, T.D., Miller, M.R., Packer, M.A. Changes in lipid class content and composition of *Isochrysis* sp. (T-Iso) grown in batch culture. *Aquacult. Int.* **23**, 1293-1312 (2015).
31. Mercer, P., Armenta, R.E., Developments in oil extraction from microalgae. *Eur. J. Lipid Sci. Technol.* **113**(5), 539-547 (2011).
32. Neil, G.W., Carmichael, C.A., Goepfert, T.J., Fulton, J.M., Knothe, G., Lau, C.P-L., Lindell, S. R., Mohammady, N.G-E., Van Mooy, B.A.S., Reddy, C.M. Beyond fatty acid methyl esters: expanding the renewable carbon profile with alkenones from *Isochrysis*.sp. *Energy Fuels.* **26**, 2434-2441 (2012).
33. Neil, G.W., Knothe, G., Williams, J.R., Burlow, N.P., Culler, A.R., Corliss, J.M., Carmichael, C.A., Reddy, C.M. Synthesis and analysis of an alkenone-free biodiesel from *Isochrysis* sp. *Energy Fuels.* **28**(4), 2677-2683 (2014).
34. Knothe, G. Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Process. Technol.* **86**, 1059-1070 (2005).
35. Valenzuela-Espinoza, E., Millán-Núñez, R., Núñez-Cebrero, F. Protein, carbohydrate, lipid and chlorophyll a content in *Isochrysis aff. galbana* (clone T-Iso) cultured with a low cost alternative to the f/2 medium. *Aquacult. Eng.* **25**, 207-216 (2002).
36. Kulkarni, M.G., Dalai, A.K., Bakshi, N.N. Utilization of green seed canola oil for biodiesel production. *J. Chem. Technol. Biotechnol.* **81**, 1886-1893 (2006).