

Manuscript Number: BITE-D-14-04150R2

Title: Effects of pulsed electric field treatment on enhancing lipid recovery from the microalga, *Scenedesmus*

Article Type: Short communication

Keywords: pulsed electric field, fatty acid methyl ester (FAME), lipids, isopropanol, Folch

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**Abstract:** Chloroform and methanol are superior solvents for lipid extraction from photosynthetic microorganisms, because they can overcome the resistance offered by the cell walls and membranes, but they are too toxic and expensive to use for large-scale fuel production. Biomass from the photosynthetic microalga *Scenedesmus*, subjected to a commercially available pre-treatment technology called Focused-Pulsed® (FP), yielded 3.1-fold more crude lipid and fatty acid methyl ester (FAME) after extraction with a range of solvents. FP treatment increased the FAME-to-crude-lipid ratio for all solvents, which means that the extraction of non-lipid materials was minimized, while the FAME profile itself was unchanged compared to the control. FP treatment also made it possible to use only a small proportion of chloroform and methanol, along with isopropanol, to obtain equivalent yields of lipid and FAME as with 100% chloroform plus methanol.

## Research Highlights

- Pulsed Electric field (PEF) pretreatment enhanced lipid recovery from *Scenedesmus*.
- Extraction of non-lipid materials minimized with PEF as evidenced by higher FAMES.
- Pretreatment minimized toxic solvent usage by 12-fold.

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6 1 Effects of pulsed electric field treatment on enhancing lipid  
7 2 recovery from the microalga, *Scenedesmus*  
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31 14 **Abstract**  
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35 16 Chloroform and methanol are superior solvents for lipid extraction from  
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37 17 photosynthetic microorganisms, because they can overcome the resistance offered by  
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39 18 the cell walls and membranes, but they are too toxic and expensive to use for large-  
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41 19 scale fuel production. Biomass from the photosynthetic microalga *Scenedesmus*,  
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43 20 subjected to a commercially available pre-treatment technology called Focused-  
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45 21 Pulsed® (FP), yielded 3.1-fold more crude lipid and fatty acid methyl ester (FAME)  
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47 22 after extraction with a range of solvents. FP treatment increased the FAME-to-crude-  
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49 23 lipid ratio for all solvents, which means that the extraction of non-lipid materials was  
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51 24 minimized, while the FAME profile itself was unchanged compared to the control.  
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53 25 FP treatment also made it possible to use only a small proportion of chloroform and  
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55 26 methanol, along with isopropanol, to obtain equivalent yields of lipid and FAME as  
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57 27 with 100% chloroform plus methanol.  
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5 29 Introduction  
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9 31 Photosynthetic microorganisms, i.e., algae and cyanobacteria, are capable of  
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11 32 generating lipids that can become feedstock for producing liquid fuels currently  
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13 33 generated from petroleum (Rittmann, 2008; Chisti, Y., 2007). Several species of  
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15 34 microalgae, including *Scenedesmus*, *Chlorella*, *Nannochloropsis*, and  
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17 35 *Chlamydomonas*, can fix carbon dioxide into high-density lipid inclusions that cause  
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19 36 the microalgae to have 30-60% of their cell dry weight as lipids (Liang et al., 2009;  
20  
21 37 Bondioli et al., 2012).

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24 38 Lipids occur mainly as triacylglycerols (TAGs) in algae and diacylglycerols (DAGs)  
25  
26 39 in cyanobacteria. TAGs are enclosed within intracellular oleosomes (Hu et al., 2008),  
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28 40 and DAGs are contained in intracellular thylakoid membranes (Hu et al., 2008).

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30 41 Extraction of these intracellular lipids demands that the solvent be able to penetrate  
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32 42 the cell wall and outer membranes, both of which may restrict its access (Sheng et al.,  
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34 43 2011b; Zbinden et al., 2013; Goettel et al., 2013; Dejoye et al., 2011).

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36 44 Two strategies have been evaluated to overcome resistance to solvent access: (1)  
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38 45 extracting the lipids with very strong solvents that dissolve the lipids and break down  
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40 46 the linkage between the lipids and membrane matrix, and (2) disrupting the cell's  
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42 47 protective layers through pre-treatment so that accessibility is improved for any added  
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44 48 solvent. The "gold standard" solvents are combinations of chloroform and methanol,  
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46 49 such as Folch (1:1 chloroform: methanol) and Bligh & Dyer (B&D, 1:1:0.5  
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48 50 chloroform: methanol: water). While effective, lipid extraction with chloroform and  
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50 51 methanol is infeasible for large-scale application, because these solvents are  
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52 52 hazardous materials and expensive (Zbinden et al., 2013). Moreover, these strong  
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53 solvents co-extract non-lipid components from the biomass, necessitating extensive  
54 downstream refining of the valuable fuel precursors (Sheng et al, 2011a).  
55 Recent approaches to make lipid recovery more sustainable include solvent-free  
56 extraction, such as supercritical CO<sub>2</sub>, or “green” solvents, such as hexane, ethyl  
57 acetate, and isopropanol. While circumventing environmental toxicity, these  
58 approaches have achieved comparatively lower yields, although pre-treatment has  
59 been helpful (Dejoye et al, 2011; Zbinden et al., 2013; Sheng et al., 2011a; Bligh and  
60 Dyer, 1959; Folch, 1957).  
61 Several pre-treatment techniques have been applied to improve lipid recovery through  
62 cell disruption and lysis. The goals are to make low-toxicity solvents work at least as  
63 well as the toxic solvents and to reduce the energy inputs for mixing and heating  
64 (Zbinden et al., 2013). Well-studied pre-treatment approaches for lipid extraction  
65 from photosynthetic biomass include mechanical, ultrasound, microwave, osmotic  
66 shock, enzymatic lysis, and pulsed electric fields (Sheng et al., 2011b; Zbinden et al.,  
67 2013; Goettel et al., 2013; Dejoye et al., 2011). The most recent entry applies a  
68 pulsed electric field (PEF) to disrupt biomass. This commercial technology is  
69 referred as Focused-Pulsed® (FP, OpenCEL, Atlanta, GA, <http://www.opencel.com>),  
70 and it has been documented to enhance hydrolysis and bioavailability for a range of  
71 biomass sources (Rittmann, 2008; Salerno et al., 2009). When FP is applied to disrupt  
72 biomass passed through a high-strength electrical field (> 30 kV) that is pulsed (~  
73 2000 hz), it disrupts cell membranes and walls as the electrical field interacts with  
74 phospholipids and the peptidoglycan.  
75 Initial trials with PEF treatment of cyanobacteria and microalgae demonstrated  
76 enhanced lipid recovery (Sheng et al., 2011b, Zbinden et al., 2013; Goettel et al.,

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77 2013), but solvent extraction remained the rate-limiting step. Here, a systematic study  
78 of how FP treatment disrupts *Scenedesmus* documents how disruption makes it  
79 possible to diminish significantly the use of toxic solvents without compromising  
80 lipid recovery in the form of FAMES.

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82 **Materials and methods**  
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84 **Sample procurement**  
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86 40 L of freshly harvested *Scenedesmus* spp. was obtained from a pilot-scale  
87 photobioreactor at the Arizona Center for Algal Technology and Innovation  
88 (AzCATi) located at ASU's Polytechnic campus. The *Scenedesmus* had been grown  
89 under nutrient-depleted conditions for achieving high lipid content (Hu et al, 2008).  
90 After transport to the Swette Center for Environmental Biotechnology (SCEB) on  
91 ASU's Tempe campus, the sample was subjected to FP treatment with the alpha unit  
92 at a treatment intensity of 30.6 KWh/m<sup>3</sup>; this is called 1-pass treatment (Salerno et al,  
93 2009). A portion of the sample that was collected from AzCATi was not subjected to  
94 treatment and was the control sample. The treated biomass (stored overnight at 4°C)  
95 was again passed through the FP unit to achieve 2-pass treatment. The second  
96 treatment achieved a treatment intensity of 33.7 KWh/m<sup>3</sup>. Overnight cooling prior to  
97 the second pass ensured that cell lysis was not caused by a temperature increase. The  
98 temperature increased from 24°C to 54 °C after 1-pass treatment, while 2-pass  
99 treatment increased the temperature from 13.5°C to 36°C. Dry weight was measured  
100 as total suspended solids (TSS), and the organic fraction of the dry weight was  
101 assayed as volatile suspended solids (VSS) according to **Standard Methods** (Rice et  
102 al, 2012). Total and semi-soluble chemical oxygen demand (TCOD and ssCOD) was  
103 assayed using HACH kits and quantification by absorbance at a wavelength of 620

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104 nm. Semi-soluble COD was obtained after filtering the sample through a 1.2- $\mu$ m  
105 glass filter (Salerno et al. 2009).

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107 **Flow Cytometry**

108 Flow cytometry measurement (FCM) of SYTOX Green-stained samples was  
109 performed using a BD FACSAria (BD Biosciences, CA, USA) flow cytometer.  
110 When cell walls were compromised by FP, SYTOX molecules were able to penetrate  
111 the cells and exhibit their characteristic green fluorescence upon staining the DNAs.  
112 The SYTOX was applied according to manufacturer guidelines (Invitrogen, Carlsbad,  
113 CA). Excitation was with an air-cooled 20 mW argon ion laser at 488 nm, and the  
114 fluorescence emission of SYTOX was detected using a 510-550 nm FITC filter with  
115 readings counted for 10,000 events from each sample. The percentages of total  
116 SYTOX stained cells were reported in Table 1, which corresponds to green  
117 fluorescent (dead/ inactivated) cells.

118 **Crude Lipids and FAME extraction by standard solvent mixtures**  
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120 About 15 g (dry weight) of control and FP-treated *Scenedesmus* biomass was freeze-  
121 dried using a FreeZone Benchtop instrument (Labconco, MO, USA). Lipid extraction  
122 followed the protocol of Sheng et al. (2011a). The solvents were Bligh and Dyer  
123 (chloroform: methanol: water = 1:2:0.8, v/v), Folch (chloroform: methanol = 2:1,  
124 V/V), hexane, and isopropanol. The solvent-to-biomass ratio was 1: 5 (v/w) for all  
125 the methods, all extractions were carried out twice, and all analyses were performed  
126 in duplicate. The mixtures were vortexed for 3 hours using a vortex mixer (Scientific  
127 Industries, NY, USA) at room temperature. After the sample was filtered through a  
128 0.2- $\mu$ m PVDF membrane (Pall Science, NY, USA) to remove the biomass debris, the

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4 129 crude lipids were dried in the filtrate in a Nitrogen evaporator (Labconco RapVap,  
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7 130 MO, USA). The crude lipid weight was obtained by subtracting the total dried weight  
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10 131 from the weight of the empty tubes and the weight of any breakthrough materials  
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12 132 released from the syringe filter when the solvents alone were passed through. The  
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14 133 statistical differences of crude-lipid and FAME recovery between control and FP pre-  
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16 134 treatment were evaluated using the Independent-Samples t-test by SPSS 22 (IBM,  
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19 135 Armonk, New York) for the cases of different solvents, solvent mixtures, and kinetic  
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22 136 extraction.

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26 137 Trans-esterification of dried crude lipid was performed by adding 2 ml of 3-N  
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28 138 methanolic HCl (Sigma-Aldrich, MO, USA) to the entire dried lipid in a test tube and  
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31 139 incubated the mixture at 85 °C in the oven for 2.5 h (Sheng et al., 2011a). For direct  
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34 140 trans-esterification, 2 mL of 3-N methanolic HCl was added to 15 mg of freeze dried  
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36 141 biomass in a test tube and incubating the mixture under similar conditions as for  
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39 142 regular trans-esterification. After cooling the mixture to room temperature, 0.5 ml DI  
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42 143 water and 1.55 ml hexane were added, the mixture was vortexed to extract the FAME  
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44 144 components, and then the 1.5-ml volumes of hexane were pooled for FAME analysis.  
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46 145 The FAME components were quantified using a gas chromatograph (Shimadzu GC  
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49 146 2010, Japan) equipped with a Supelco SP-2380 capillary column (30 m x 0.25 mm x  
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51 147 0.20 µm) and flame ionization detector (FID). The outputs were calibrated against a  
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54 148 37-Component FAME Mix standard (Supelco, PA, USA).

#### 55 56 57 58 149 Crude Lipids and FAME extraction with solvent mixtures 59 60 150

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62 151 Different volume ratios of the Folch solvent and isopropanol were tested on the same  
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65 152 samples of control and FP-treated biomass. Maintaining the total solvent volume at 3  
153 mL, the Folch: Isopropanol (% by volume) ratio was varied as follows: 0, 3.3, 8.3,



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4 154 16.7, 33.3, 66.7 and 100%. The extraction performance at each ratio was evaluated in  
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7 155 terms of crude lipids and FAME content. The crude lipid weight was obtained  
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10 156 following the method mentioned above.

### 13 157 Effect of vortex time on crude lipids and FAMEs extraction efficiency

15 158 The effect of vortexing time as a measure of the energy input needed to achieve a  
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18 159 target extraction efficiency was evaluated. Extraction efficiency for control- and FP-  
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20 160 treated biomass was evaluated with vortexing times of 0.5, 1, 2, and 4 minutes, after  
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23 161 which crude lipids and FAME contents were evaluated using extraction with 100%  
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26 162 Folch solvent.

## 30 163 Results and discussion

### 32 164 Sample characterization before and after FP treatment

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37 166 Table 1 summarizes how FP treatment affected key physical and chemical  
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39 167 characteristic of *Scenedesmus* biomass. TSS and VSS were almost unchanged by FP-  
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42 168 treatment; this is consistent with past work on other types of biomass (Sheng et al.,  
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44 169 2011b; Salerno et al, 2009) and underscores that FP treatment disrupts the biomass  
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47 170 instead of destroying it. One-pass FP treatment increased the concentration of ssCOD  
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50 171 by 54%, but the second pass increased ssCOD by only another 9% (data not shown).  
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52 172 The increases to ssCOD were substantially larger than for *Synechocystis* PCC 6803  
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55 173 cells for similar treatment intensity (Sheng et al., 2011b), which was only 5%. The  
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57 174 pH decreased after FP treatment, probably due to the release of soluble fatty acids  
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60 175 (Chen et al., 2012).

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4 178 Flow Cytometry  
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9 180 Flow cytometry with the SYTOX stain gauged the efficiency of cell lysis by FP  
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11 181 pretreatment. The green fluorescence intensity increased by several orders of  
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13 182 magnitude for 1-Pass (from up to  $10^3$  to  $10^5$  units). In addition, the fraction of stained  
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16 183 (inactive) cells increased dramatically after FP treatment: from 5% in the control to  
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18 184 97% (as shown in Table 1).

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22 185 Lipid and FAME recovery  
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27 187 Figure 1 shows that the lipid recovery associated with FP treatment and different  
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29 188 solvents. Compared with control biomass, FP treatment improved crude-lipid  
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32 189 recovery by about 47, 71, 78, and 90% for B&D, Folch, hexane, and isopropanol,  
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34 190 respectively. The solvent-extraction performance followed a similar order similar to  
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37 191 what has been reported in the literature: Folch > B&D >> hexane  $\geq$  isopropanol  
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39 192 (Sheng et al., 2011a; Keris-Sen et al., 2014). The impact of FP treatment was even  
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41 193 greater for FAME: as much as a 310% increase for hexane.

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45 194 FAME recovery was always lower than crude lipid recovery for all solvents,  
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47 195 indicating co-extraction of non-lipid components, like protein, carbohydrate, and  
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50 196 pigment (Laurens et al., 2012). Several combinations of isopropanol and Folch  
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52 197 solvents following FP treatment yielded the maximum FAME-to-biomass ratio,  
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55 198 around 21% (Fig 1b). FP treatment improved accessibility of these solvents to the  
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58 199 FAME targets rather than non-FAME materials, and the FAME: crude lipid ratio  
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60 200 increased. In addition, direct transesterification of the untreated biomass yielded total  
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63 201 FAME of 21.5 3.4%, implying that FP treatment with the best combinations of  
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65 202 solvent extraction could achieve ~100% of the maximum extractable FAME.

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4 203 Solvent requirement reduced by FP treatment  
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6 204 The Folch solvent plays an important role in solubilizing lipids and liberating the  
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9 205 bound lipids from the membrane matrix. Figure 2 shows that extracted crude lipids  
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11 206 and FAME increased with an increasing volume ratio of Folch solvent in Folch +  
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14 207 isopropanol mixtures. A clear advantage of using FP treatment is that it reduced the  
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16 208 amount of Folch solvent needed to obtain an equivalent FAME yield. For FP-treated  
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19 209 biomass, the FAME yield obtained by adding 66.7% Folch was similar to the FAME  
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21 210 yield obtained by extraction with 100% Folch. Even more importantly, the FAME  
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24 211 yield obtained from FP-treated biomass using only 8.3% Folch was higher than the  
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27 212 FAME yield obtained from control biomass by using 100% Folch solvent. Therefore,  
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29 213 FP treatment significantly reduced the need for toxic Folch solvent (~12-fold) to get  
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32 214 an equivalent yield of FAMEs from control *Scenedesmus* biomass. FAME profiles  
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34 215 (%) were similar for all conditions, which confirm that FP treatment did not modify  
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37 216 the inherent FAME composition and it mainly helped to improve the extraction  
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39 217 efficiency. In fact, increasing Folch solvent with FP treatment diluted the benefit due  
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42 218 to a decline in the FAME-to-crude lipids ratio. Thus, an optimum solvent dosage for  
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45 219 FAME recovery after FP treatment was achieved.

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48 220 In addition, Figure 3 shows that FP treatment reduced the vortex time by almost two  
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51 221 orders of magnitude to achieve the same recovery of crude lipids and FAME. FP-  
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53 222 treated biomass gave nearly the same FAME-recovery efficiency after 2 minutes of  
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56 223 vortex time as for the control after 3 hours of vortexing. Thus, FP treatment lowered  
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59 224 the energy input needed for mixing.

## 225 Conclusions

226 FP treatment increased the yield of FAME by as much as 3.1-fold using hexane over  
227 control *Scenedesmus*, while also increasing the FAME-to-crude-lipid ratio for all

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4 228 solvent conditions, and the FAME profile was not affected by FP treatment. Thus,  
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7 229 extraction generated more of the truly useful fatty acids for biofuel production after  
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10 230 the *Scenedesmus* biomass was treated by FP. FP treatment also reduced the usage of  
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12 231 toxic solvents (chloroform and methanol) by 12-fold for equivalent yields of lipid and  
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14 232 FAME and significantly lowered the mixing energy requirements. Thus, FP treatment  
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17 233 provides a sustainable strategy for extracting fuel feedstock from photosynthetic  
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20 234 microorganisms.

## 235 Acknowledgements

236 The project was sponsored by LightWorks, Arizona State University. We would also  
27  
28 237 like to thank Mr. Jared Alder of OpenCEL/Trojan Technologies for assisting with the  
29  
30  
31 238 alpha unit operation. We thank Dr. John McGowen and the Arizona Center for Algal  
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33 239 Technology and Innovation (AzCATi) for generously supplying algal biomass. We  
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36 240 thank Mr. David Lowry at the Electron microscopy facility at the School of Life  
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38 241 Sciences (SoLS) at Arizona State University with his expertise in sample preparation  
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41 242 and use of the TEM.

## 243 Supporting Information

244 The supporting information contains 8 pages, with four sections: Transmission  
48  
49 245 Electron Microscopy, Cell Lysis Evaluation by Flow Cytometer, Particle Size  
50  
51 246 Analysis, FAME-to-crude lipid ratios, and the FAMEs profile for all solvents and pre-  
52  
53  
54 247 treatment conditions. This includes Figures S1 to S5 and Table S1.

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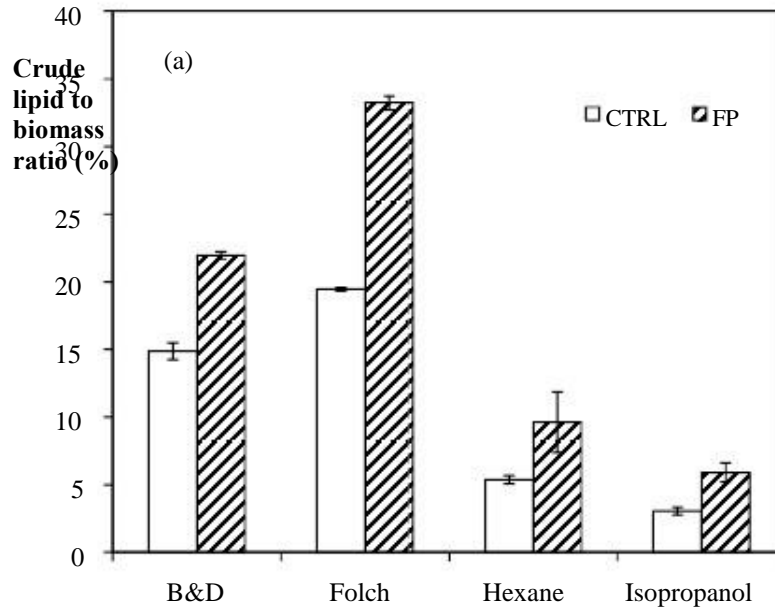
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307 **Table 1** Summary of physical and chemical parameters of *Scenedesmus* biomass  
308 before and after FP treatment

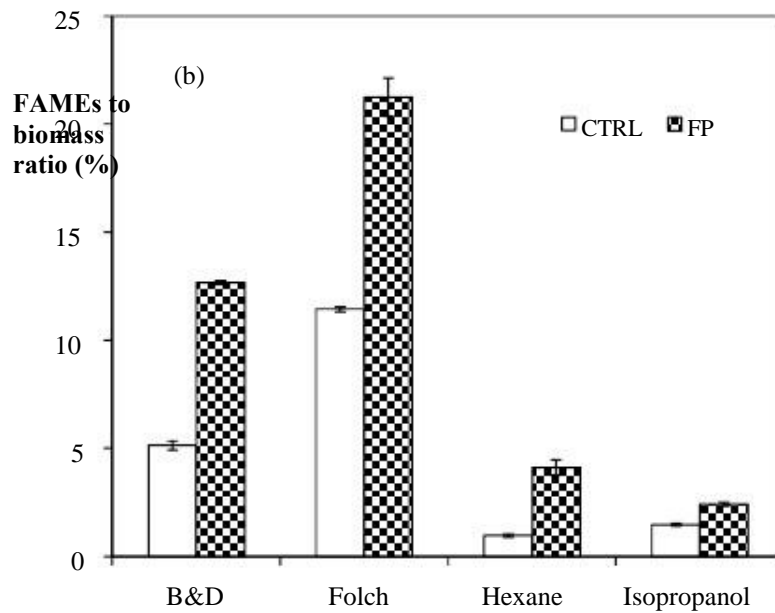
	Control	FP_1 pass
Treatment intensity (Kwh/m <sup>3</sup> )	--	30.6
Temperature change	24°C	26->53°C
pH	7.42	6.97
TSS (mg/L)	4600±40	4440±30
VSS (mg/L)	4470±50	4300±30
TCOD (mg/L)	8000±30	8000±60
ssCOD (mg/L)	450±10	690±10
Increased ssCOD (% to control)		50
% of total particles stained with SYTOX#	4.7	96.8

#10,000 cell counting events;

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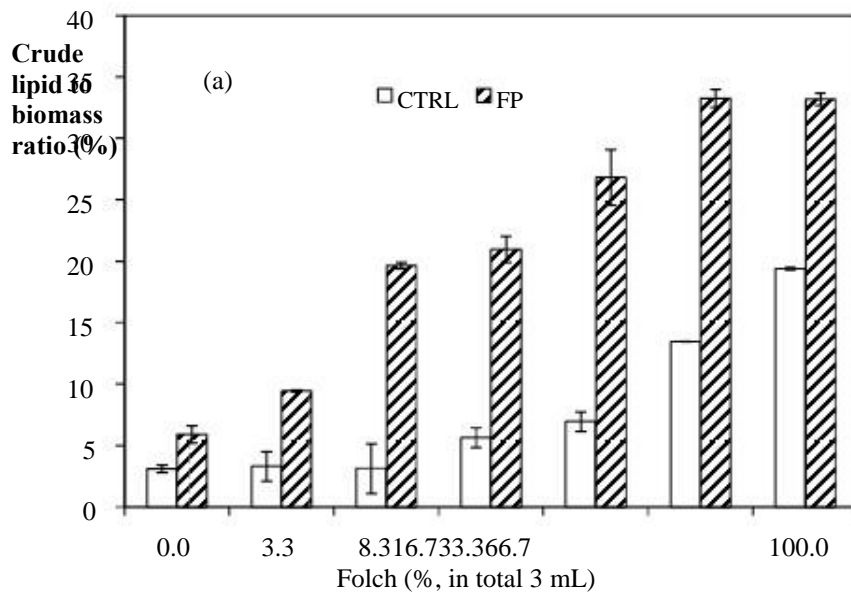


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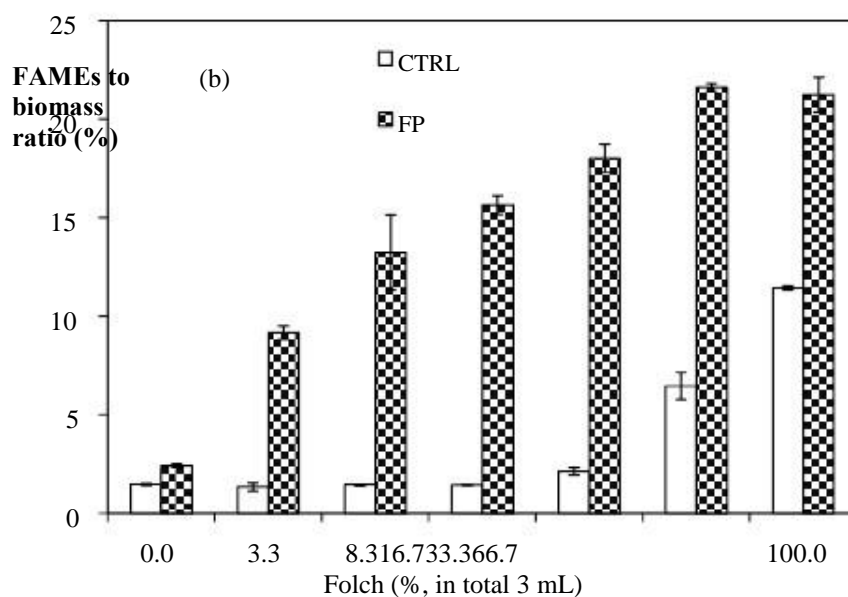
318 **Figure 1** Crude lipid (a) and FAME (b) recoveries (% of dry weight) for four solvent  
 319 systems -- Bligh and Dyer (B&D), Folch, hexane, and isopropanol -- for control and  
 320 FP-treated *Scenedesmus* biomass (1\_pass) samples. Results for 2\_pass samples were  
 321 similar and are not shown. The difference of FAME recovery was significant  
 322 between CTRL and FP within the group of the same solvent ( $P < 0.05$ ).

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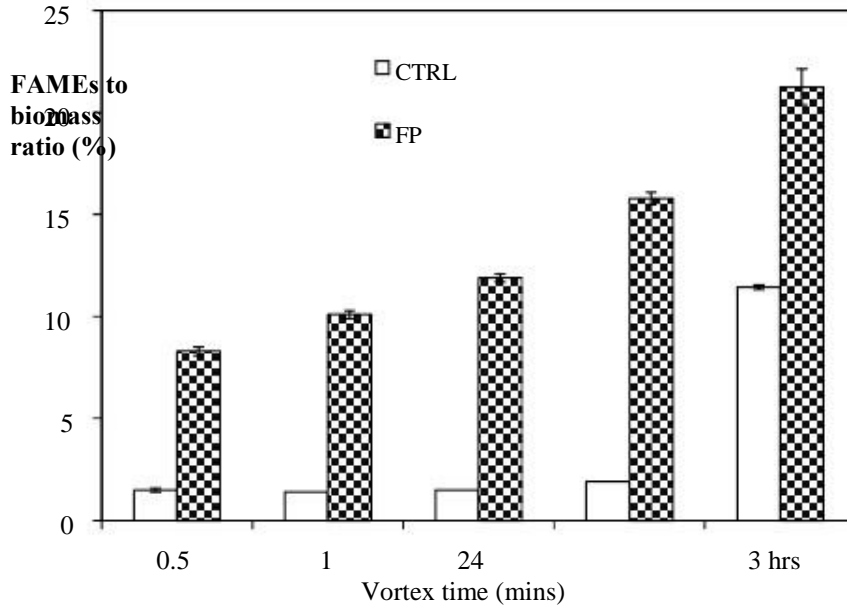
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327 Figure 2 Crude lipid (a) and FAME (b) recoveries (% of dry weight) for different  
 328 ratios of Folch and isopropanol solvent combinations with ratios (% by volume) for  
 329 control and 1-pass FP-treated *Scenedesmus* biomass. The difference of FAME  
 330 recovery was significant between CTRL and FP within the group of the same solvent  
 331 ( $P < 0.05$ ).  
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336 Figure 3 FAME recovery (% of dry weight) with different vortexing times for  
 337 Control and 1-pass FP-treated *Scenedesmus* and using 100% Folch solvent. The  
 338 difference of FAME recovery was significant between CTRL and FP within the same  
 339 duration time of vortex ( $P < 0.05$ ).  
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**Electronic Annex**

**[Click here to download Electronic Annex: Supporting Information\\_Solvent Extraction\\_Re-revision 092214 final.docx](#)**