Assessment of biomass potentials of microalgal communities in open pond raceways using mass cultivation

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34 **Abstract**

35 Metagenome studies have provided us with insight into the complex interactions of
36 microorganisms with their environments and hosts. Few studies have focused on microalgae-
37 associated metagenomes, and no study has addressed aquatic microalgae and their bacterial
38 communities in open pond raceways (OPRs). This study explored the possibility of using
39 microalgal biomasses from OPRs for biodiesel and biofertilizer production. The fatty acid profiles
40 of the biomasses and the physical and chemical properties of derived fuels were evaluated. In
41 addition, the phenotype-based environmental adaptation ability of soybean plants was assessed.
42 The growth rate, biomass, and lipid productivity of microalgae were also examined during mass
43 cultivation from April to November 2017. Metagenomics analysis using MiSeq identified ~127
44 eukaryotic phylotypes following mass cultivation with (OPR 1) or without (OPR 3) a
45 semitransparent film. Of these, ~80 phylotypes were found in both OPRs, while 23 and 24
46 phylotypes were identified in OPRs 1 and 3, respectively. The phylotypes belonged to various
47 genera, such as *Desmodesmus*, *Pseudopediastrum*, *Tetradesmus*, and *Chlorella*, of which, the
48 dominant microalgal species was *Desmodesmus* sp.. On average, OPRs 1 and 3 produced ~8.6 and
49 9.9 g·m^{-2}·d^{-1} (0.307 and 0.309 DW·L^{-1}) of total biomass, respectively, of which 14.0 and 13.3
50 wt% respectively, was lipid content. Fatty acid profiling revealed that total saturated fatty acids
51 (mainly C16:0) of biodiesel obtained from the microalgal biomasses in OPRs 1 and 3 were 34.93%
52 and 32.85%, respectively; total monounsaturated fatty acids (C16:1 and C18:1) were 32.40% and
53 31.64%, respectively; and polyunsaturated fatty acids (including C18:3) were 32.68% and 35.50%,
54 respectively. Fuel properties determined by empirical equations were within the limits of biodiesel
55 standards ASTM D6751 and EN 14214. Culture solutions with or without microalgal biomasses
56 enhanced the environmental adaptation ability of soybean plants, increasing their seed production.
57 Therefore, microalgal biomass produced through mass cultivation is excellent feedstock for
58 producing high-quality biodiesel and biofertilizer.

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Introduction

Microalgae, which have evolved over a long period, are biologically diverse organisms that are highly valuable for academic and industrial fields. Microalgae have high cellular lipid content and so have distinct characteristics that may be used for biodiesel production or for the production of various other useful substances, such as valuable unsaturated fatty acids (UFAs), proteins, vitamins, and carotenoid secondary metabolites (Ren et al., 2017). Therefore, microalgae are increasingly used to produce high-value products and bioactive substances in a wide spectrum of fields ranging from sustainable energy to healthcare (Jebali et al., 2019). Global microalgal research can be considered the next-generation industry that will spearhead the growth of a green and low-carbon era (Bell et al., 2016).

Microalgae can generate large amounts of carbon-rich fatty acids from CO_2 and grow fast in poor-quality waters or on non-arable lands without depleting food resources, so they are effective biofuel sources (Simas-Rodrigues et al., 2015; Faried et al., 2017; Ren et al., 2017). Microalgal strains of industrial value are found by either characterizing natural strains or establishing artificial strains through genetic manipulation. Isolating of new microalgal strains from the environment enables the finding of new strains that have unique physiological features and are promising biofuel sources (Abou-Shanab et al., 2011). This approach is effective because of myriads of uncharacterized microalgal strains in nature. However, artificial strain improvement through genetic engineering depends exclusively on the microalgal genetic information available (Cheng et al., 2019). Unfortunately, despite the industrial importance of microalgae, research on microalgal genetic engineering is limited compared to similar research on other organisms (Ng et al., 2017). Microalgal genetic engineering technologies have been fully established only for *Chlamydomonas reinhardtii* (Young & Purton, 2016). Although other microalgal strains have been successfully transformed, these are usually one-time cases with low reproducibility (Siddiqui et al., 2019). In addition, native isolates are well adapted to local conditions and exhibit better performance and robustness (Alishah Aratboni et al., 2019). Therefore, for large-scale industrial uses of microalgae, it is necessary to establish a microalgal mass cultivation system, such as the open pond raceway (OPR), using indigenous microalgal species instead of engineered strains.

To realize the potential of microalgae, improvements are required in cost, productivity, and sustainability (Dahlin et al., 2018). To scale up production at lower costs, open pond systems coupled with fresh water or wastewater are a viable alternative (Bell et al., 2016). These systems

97 are operated in semi-continuous or continuous mode to meet industrial production standards by

98 maximizing both biomass and lipid productivities, while ensuring the industrial suitability of the

99 microalga-derived fatty acids (Song et al., 2013). However, although outdoor productivity is often

100 less than the laboratory yield because of the extreme dynamics of environmental conditions, such

101 as water temperature and light intensity, studies on identifying optimal strains have rarely

102 investigated these criteria in semi-continuous or continuous cultures (Dahlin et al., 2018).

103 Recently, several studies questioned the sustainability of biofuel production from freshwater

104 species because itis uneconomical (Chisti, 2013). Because of these limitations, most studies on

105 microalgae are conducted in closed photobioreactors (Ren et al., 2017). However, an open pond

106 system is still needed in order to produce bioenergy as an alternative to fossil fuel.

107   In industrial-scale open pond cultivation with fresh water, many environmental (light and

108 temperature), operational (pH, CO_2 and nutrients), and biological (bacteria and fungi) parameters

109 affect microalgal diversity and productivity, in turn affecting biomass quality and quantity (Cho et

110 al., 2015). Therefore, open pond systems are prone to colonization by environmental microbes and

111 may contain numerous distinct taxa (El-Sheekh et al., 2019). In the microalgal-based biofuel field,

112 maintaining the integrity of the dominant microalgal species is mandatory, so there is a lot of

113 uncertainty around biofuel production through freshwater systems (Roccuzzo, Beckerman &

114 Pandhal, 2016). In addition, the feasibility of maintaining dominant microalgal communities in

115 fresh water-dependent ponds and the factors involved thereof are unclear (Park, Craggs & Shilton,

116 2013). Undesirable colonization can be controlled by using locally adapted microalgae observed

117 in natural ecosystems, and extensive studies have been conducted to probe locally isolated

118 microalgae strains for their potential to be used in biofuel production (Rodolfi et al., 2009).

119   Metagenome technologies are powerful tools for analyzing complex microbial communities

120 and have led to a tremendous increase in the knowledge of the functions, protein families,

121 biotechnology, and ecology of microbial communities (Hugenholtz et al., 1998; Krohn-Molt et al.,

122 2017). Metagenome studies have provided us with insight into the complex interactions of

123 microorganisms with their environments and hosts (Krohn-Molt et al., 2017). Surprisingly, few

124 studies have focused on microalgae-associated metagenomes (Krohn-Molt et al., 2017; Sambles

125 et al., 2017), and no study has addressed aquatic microalgae and their bacterial communities in

126 OPRs. However, the microalgal community based on phylogenetic and metagenomic diversity is

127 growing.

128   This study investigated the effect of various factors on the diversity and productivity of an
129   indigenous microalgal community in OPRs with (OPR 1) or without (OPR 3) a semitransparent
130   film through three seasons. We modeled the biodiversity of a microalgal mass cultivation system
131   by considering environmental factors, such as water temperature, intra- and interspecies
132   competition in the microalgal community, and the abundance of other species, including
133   cyanobacteria. We measured biomass-based lipid production in fresh water dependent OPRs
134   operated during mass cultivation from April to November 2017. To identify locally adapted
135   microalgal strains, we examined and statistically analyzed various environmental (meteorological
136   and nutritional), biological (algal and cyanobacterial diversity), and physiochemical (fatty acid
137   profile and biodiesel quality) parameters. Our findings may facilitate biofuel and biofertilizer
138   production from freshwater system biomasses.
139

Materials and methods

Operation of an OPR system

142   A microalgal semi-continuous mass cultivation system comprising two OPRs (~675.0 m^2) with
143   (OPR 1) or without (OPR 3) a semitransparent film at Chilgok-gun Agricultural Technology
144   Center (36° 02′ 18.91″ N, 128° 22′ 57.7″ E), Korea, was operated to monitor the ecological system
145   and biodiversity in microalgae-dependent mode under natural environmental conditions. The mass
146   cultivation period was from April 1 to November 30, 2017. OPRs used natural sunlight. We
147   supplemented 236 tons of groundwater with Eco-Sol (nitrogen:phosphorus:potassium = 25:9:18;
148   Farm Hannong, Ulsan, Korea) as a commercial water-soluble fertilizer to produce a final
149   concentration of 10.0–18.0 mg·L^{-1} total nitrogen (TN) and 2.0–3.0 mg·L^{-1} total phosphorus (TP).
150   Next, we injected 10% of CO_2 in air at a flow rate of 10 L·min^{-1} in both OPRs using a venturi
151   system during daylight hours (Hong et al., 2017). The OPR temperature and pH were measured
152   every 3 h using a WQC-24 portable multiparameter water quality meter (Multi Measuring
153   Instruments Co. Ltd., Tokyo, Japan).
154

Microalgae mass cultivation

156   Indigenous microalgae from earlier cultivation periods were used. Briefly, *Desmodesmus* sp., the
157   dominant species grown in OPRs, was inoculated, and species succession was monitored at
158   morphological and molecular levels. Semi-continuous cultivation was conducted at a velocity of

159 25‑30 cm·s⁻¹. Next, we harvested 155 tons (approximately two-thirds of the microalgal culture

160 from each raceway and replaced it with the same volume of underground water with appropriate

161 nutrient levels; the remaining culture was used as a seed. The mass cultivation was continuously

162 recycled throughout the study. When TN and TP were almost depleted and the optical density

163 (OD) and total biomass reached their maximum values, the culture was harvested. TN and TP

164 levels were quantitated using HS-TN(CA)-L and HS-TP-L water test kits (Humas, Daejeon,

165 Korea), and OD was measured using a spectrophotometer (X-ma 1200V; Human Corp., Seoul,

166 Korea) at a wavelength of at 680 nm. The total biomass was dried in a dry oven and weighed.

167 Finally, to observe morphological changes, the microalgal cultures were sampled every 3 days and

168 inspected at 400 × magnification under an Eclipse E100 microscope (Nikon Instruments Inc.,

169 Tokyo, Japan).

170

171 **MiSeq-based microalgal diversity**

172 To determine mock communities, we screened OPRs for the presence of *Acutodesmus* sp.

173 KNUA038, *Chlamydomonas* sp. KNUA023, *Chlamydomonas* sp. KNUA040, *Chlorella* sp.

174 KNUA027, *Desmodesmus* sp. KNUA024, *Pseudopediastrum* sp. KNUA039, and *Scenedesmus*

175 *obliquus* KNUA019 by sequencing 18S ribosomal RNA(rRNA) V4 and V8–V9 regions, as

176 previously described (Bradley, Ian M, Pinto, Ameet J, Guest, 2016). We also confirmed microalgal

177 identification by using the Basic Local Alignment Search Tool (BLAST) against the National

178 Center for Biotechnology Information (NCBI) GenBank database.

179   Next, we extracted genomic DNA (gDNA) from the harvested biomass using a DNeasy

180 Plant Mini kit (Qiagen, Hilden, Germany) and purified it using a Wizard DNA Clean-Up System

181 (Promega, Madison, WI, USA) according to the manufacturers' instructions. Then, 16S and 18S

182 rRNA genes were used to identify cyanobacteria and microalgae, respectively, within the OPRs.

183 Each sequencing sample was prepared in accordance with Illumina 16S and 18S metagenomic

184 sequencing library protocols, as previously described (de Muinck et al., 2017; Kim et al., 2017).

185 Briefly, for 16S rRNA, the internal transcribed spacer 2 (ITS2) region was amplified using PCR

186 with the ITS3/ITS4 primer set (de Muinck et al., 2017; Kim et al., 2017). The 18S protocol was

187 designed to broadly recognize eukaryotes, favoring microbial eukaryotic lineages. The primers

188 were based on a previous report (Amaral-Zettler et al., 2009) and designed to be used with the

189 Illumina MiSeq platform (Bradley, Ian M, Pinto, Ameet J, Guest, 2016). The 18S libraries on

190   MiSeq were run, as previously described (Caporaso et al., 2012), and PCR amplicons derived from

191   the conserved V8–V9 region by using the Mi1422F/Mi1510R primer pair were sequenced. Table

192   S1 shows the primer sets overhanging the pre-adapter and sequencing adapter. The outlines of the

193   16S and 18S protocols were the same. Sequences were processed and data mining performed, as

194   previously described (Kozich et al., 2013; Bradley, Ian M, Pinto, Ameet J, Guest, 2016). Finally,

195   the consensus taxonomy of the operational taxonomic unit (OTU) was constructed using Silva

196   v119 taxonomy information (Ondov, Bergman & Phillippy, 2011).

197

198   **Microalgal biomass characterization**

199   First, the harvested biomass was freeze-dried, pulverized using a mortar and pestle, and sieved

200   through an ASTM No. 230 mesh (pore size = 63 μm). Next, the total lipid content was determined

201   using a the sulfo-phospho-vanillin colorimetric method, as previously described with

202   modifications (Mishra et al., 2014). Proximate analysis was performed to measure ash content

203   using a DTG-60A thermal analyzer (Shimadzu, Kyoto, Japan) and platinum pans containing ~10

204   mg of each sample or 30 mg of α-alumina (α-Al_2O_3) powder (Shimadzu) as a reference. Nitrogen

205   (> 99.999%, N_2) was supplied as the carrier gas at a rate of 25 mL·min^{-1} to prevent oxidation of

206   the microalgae powder. The samples were heated from 50 °C to 900 °C at a rate of 10 °C·min^{-1}.

207   Thermogravimetric analysis (TGA) data were obtained using ta60 ver. 2.21 software (Shimadzu)

208   according to the manufacturer's instructions. Ultimate analysis was performed using a Flash 2000

209   elemental analyzer (Thermo Fisher Scientific, Milan, Italy) to determine the carbon (C), hydrogen

210   (H), nitrogen (N), and sulfur (S) content, and the oxygen (O) content was calculated by subtracting

211   the ash and CHNS content from the total. The calorific value (CV, also known as higher heating

212   value [HHV]) was estimated using the following equation developed by Friedl et al. (2005):

213

214   $$CV = \left\{ 3.55C^2 - 232C - 2{,}230H + 51.2C \times H + 131N + 20{,}600 \ (MJ/kg) \right\}$$

215

216   **Gas chromatography-mass spectrometry analysis**

217   Lipid extraction was performed as previously described (Breuer et al., 2013). The fatty acid methyl

218   ester (FAME) composition was analyzed using a 7890A gas chromatograph equipped with a

219   5975C mass selective detector (Agilent, Santa Clara, CA, USA). Gas chromatography (GC) runs

220   were performed using a DB-FFAP column (30 m, 250 μm ID, 0.25 μm film thickness; Agilent).

221 The initial oven temperature of the gas chromatograph was set to 50 °C and maintained for 1 min;
222 subsequently, it was increased first to 200 °C at a rate of 10 °C·min⁻¹ for 30 min and then to 240
223 °C at a rate of 10 °C·min⁻¹, which was maintained for 20 min. The injection volume was 1 µL,
224 with a split ratio of 20: 1. Helium was used as a carrier gas at a constant flow rate of 1 mL min⁻¹.
225 The mass spectrometry (MS) parameters were as follows: injector and source temperatures of 250
226 °C and 230 °C, respectively; electron impact mode at an acceleration voltage of 70 eV for sample
227 ionization; and acquisition range of 50–550 *m/z*. Compound identification was performed by
228 matching the mass spectra with those in Wiley/NBS libraries; matches > 90% were considered
229 valid.

230

231 **Biodiesel quality assessment**
232 The following physical parameters were measured to assess biodiesel quality: saponification value
233 (SV), iodine value (IV), degree of unsaturation (DU), cetane number (CN), long-chain saturated
234 factor (LCSF), and cold filter plugging point (CFPP). The parameters were quantitated on the basis
235 of the fatty acid compositions using the following empirical equations, as previously described
236 (Ramos et al., 2009):

237

238
$$SV = \Sigma \frac{560 \times N}{M}$$

239
$$IV = \Sigma \frac{254 \times N \times D}{M}$$

240
$$CN = \left(46.3 + \frac{5458}{SV}\right) - \left(0.225 \times IV\right)$$

241
$$LCSF = \{0.1 \times N(C16:0)\} + \{0.5 \times N(C18:0)\} +$$
242
$$\{1 \times N(C20:0)\} + \{1.5 \times N(C22:0)\} + \{2 \times N(C24:0)\}$$

243
$$CFPP = (3.1417 \times LCSF) - 16.477$$

244
$$DU = MUFA + (2 \times PUFA)$$

245

246 where *D*, *M*, and *N* denote the number of double bonds, average molecular mass, and weight
247 percent (wt%) of each fatty acid, respectively; MUFA refers to monounsaturated fatty acid; and
248 PUFA refers to polyunsaturated fatty acid. C16:0, C18:0, C20:0, and C22:0 are also in wt%.

249

250 **Biological enhancer assay in soybean plants**

251   First, soybean seeds (Daewon) were sown in cultivation soil, germinated at 30 °C for ~7 days in a

252   growth chamber (70–100 µmol m^{-2}·s^{-1}) in a 14 h/10 h dark cycle, at 60% humidity, and grown to

253   the V2 stage in the same incubator. Next, 15 soybean seedlings were transplanted to a large pot 30

254   cm in diameter and then cultivated near a mass culture open pond system. Subsequently, we

255   applied the following treatments to the soybeans, with the solutions applied every 3 days:

256

257   1. The control pot was supplemented with 1 L of groundwater.

258   2. The experimental pot was supplemented with 1 L of the eco-sol medium as a soil drench.

259   3. The supernatant (1 L) of the microalgal culture was collected by centrifugation at 4,000 rpm

260   for 20 min at 20 °C and mixed with the eco-sol medium as a soil drench.

261   4. The culture solution (1 L) containing microalgal biomass and the supernatant as a soil drench.

262   5. The pot with soybean plants was sprayed with 1 L of microalga-free supernatant.

263

264   The soybean plants' environmental adaptation was assessed by monitoring the plant phenotype,

265   and soybean productivity was measured by examining seed maturation and the pod weight per

266   seedling.

267

268   **Statistical analysis**

269   All experiments were performed in at least triplicate. Data were shown as the average of triplicates,

270   and error bars represented the standard deviation. $P < 0.05$ was considered statistically significant.

271

272   # Results

273   **Biodiversity of mass cultivation in open pond raceways**

274   *Establishment of MiSeq-based qualitative verification*

275   First, we examined the reported primer sets to determine whether the microalgal communitiy

276   comprising the seven known microalgal species (*Acutodesmus* sp. KNUA038, *Chlamydomonas*

277   sp. KNUA023, *Chlamydomonas* sp. KNUA040, *Chlorella* sp. KNUA027, *Desmodesmus* sp.

278   KNUA024, *Pseudopediastrum* sp. KNUA039, and *Scenedesmus obliquus* KNUA019) could be

279   effectively characterized by high-throughput sequencing (HTS). PCR amplification with primers

280   for actin, *rbcL*, and 18S rRNA V4, V8–V9, and V9 regions yielded 180–650 bp amplicons from

281   the pooled gDNA isolated analyzed samples (Fig. S1, and Data S1). We obtained an amplicon of

282   ~610 bp with *rbcL* primers 28S2F/28S2R (Fig. S1B). However, no amplicon was detected in

283   analyzed samples with actin primers (Fig. S1A).

284   Next, we introduced various regions of the 18S rRNA gene into HTS-based comparative

285   analysis. Of them, V4 and V8–V9 regions were significant, and the taxonomic groups for the V8–

286   V9 region showed higher concordance rates compared to the V4 region. The V4 region failed to

287   reliably capture two of the seven microalgal species included in this study (*Acutodesmus* sp.

288   KNUA0038 [accession no. KT883908] and *Scenedesmus* sp. KNUA019 [unregistered]), while the

289   V8–V9 region accurately represented the mean relative abundance (Tables S2 and S3). However,

290   the V9 region was too short (< 200 nucleotides [nt]) to allow overlap between forward and reverse

291   reads in the Illumina Miseq platform (250–300-nt single read length, resulting in 450‑500-nt-long

292   combined reads with 50–150 bp overlaps) (Bradley, Ian M, Pinto, Ameet J, Guest, 2016). Results

293   showed that the V8–V9 region of the 18S rRNA gene is more effective for HTS compared to other

294   candidate genes because 400–500 bp amplicons are required. Figure S2A shows schematic

295   diagrams showing the biodiversity analysis.

296

297   *Changes in environmental factors and growth parameter during open-pond mass cultivation*

298   We analyzed environmental conditions in OPRs 1 and 3 during mass cultivation from April to

299   November 2017 because the water surface was frozen in OPR 3 (without a semitransparent film)

300   from December 2017 to February 2018 but not in OPR 1 (with the same semitransparent film)

301   (Fig. S2B and Data S2). The mean water temperature in spring (April–May), summer (June–

302   August), and the fall (September–November), was 17.4 °C, 25.4 °C, and 14.1 °C, respectively

303   (Fig. 1A). Although we observed no differences between OPRs 1 and 3, the temperature of OPR

304   1 was higher compared to OPR 3 during mass cultivation, except for summer. In addition, pH was

305   neutral to heavy alkaline (7.2–10.4), becoming increasingly neutral with a gradual increase in

306   water temperature (Fig. 1B).

307   With regard to dissolved nutrients, TN and TP levels were strongly depleted in summer

308   compared to spring and the fall, concurrent with a rapid increase in total biomass and lipid levels.

309   Compared to initial TN levels (~17 mg·L^{-1}), ~13.57–16.69 mg·L^{-1} was removed in summer and

310   ~10.02–13.21 mg·L^{-1} in spring and the fall. Initial TP levels were 2.45–2.63 mg·L^{-1}, 2.35–2.62

311   mg·L^{-1} in summer, and 1.47–2.22 mg·L^{-1} in spring and the fall (Fig. 1C, and Data S2). Overall,

312   TN and TP consumption was 12.87 mg·L^{-1} (86.2%) and 2.26 mg·L^{-1} (96.3%), respectively, in

313   OPR 1 but 13.14 mg·L^{-1} (90.1%) and 2.10 mg·L^{-1} (93.5%) in OPR 3, which confirmed the higher

314   nutrient removal rate in TP compared to TN.

315   We also investigated the relationship between nutrient consumption and biomass

316   productivity. Total biomass was based on growth kinetics measured from the OD and DW. The

317   absorbance and DW of OPR #3 were higher compared to OPR 1 in June–August 2017, with the

318   highest difference in summer, when water temperature increased. OPR 3 had a maximum biomass

319   production of 17.6 g·m^{-2}·d^{-1}, while OPR 1 in the greenhouse had a maximum microalgal biomass

320   production of 13.3 g·m^{-2}·d^{-1} (Fig. 1E and Data S2). OPRs 1 and 3 had a mean total microalgal

321   biomass production of ~8.6–9.9 g·m^{-2}·d^{-1}, corresponding to 0.307–0.309 DW·L^{-1} (Figs. 1D–F and

322   Data S2).

323

324   *Overview of MiSeq-based microalgal community during mass cultivation*

325   We monitored the microalgal community using MiSeq with DNA isolated from the pooled

326   microalgal biomass during mass cultivation from April to November 2017. PCR products were

327   visualized to identify contaminated samples (Fig. S2C) and then used in MiSeq. MiSeq analysis

328   produced results for the OTU and Shannon (*H´*) and Simpson (*D*) indices (Data S3). The number

329   of OTUs in OPR 1 and 3 significantly increased in the fall compared to spring and summer,

330   although the two OPRs showed a slight difference. The *H´* index in July–November 2017 was

331   within the typical range of 1.5–3.5, as previously reported (Bradley, Ian M, Pinto, Ameet J, Guest,

332   2016), and in April–June 2017 was rarely < 1.5. During the testing period, the *D* index, the most

333   common dominance parameter, decreased with time, indicating that diversity increases with regard

334   to evenness (Fig. S3). Richness and evenness are confounded in the *H´* index, so many biodiversity

335   researchers prefer to use two indices for comparative studies, combining a direct estimate of

336   species richness (i.e., the total number of species in the community) with some measurement of

337   dominance or evenness (Caporaso et al., 2010).

338

339   *Microalgal succession during mass cultivation*

340   The following eukaryotic organisms, including microalgae, were identified in OPR 1:

341   *Desmodesmus* sp. (62%), *Heterocypris* sp. (5%), *Amoeboaphelidium* sp. (4%), *Pseudodifflugia* cf.

342   (2%), *Chlorella* sp. (1%), *Coelastrum pseudomicroporum* (1%), *Pseudopediastrum integrum*

343   (1%), *Chlamydomonadales* sp. (0.9%), *Tetradesmus obliquus* (0.8%), *Hariotina reticulate* (0.3%),

344   *Scenedesmus* sp. (0.1%), and others (18%), such as uncultured eukaryotes (Fig. 2). The following

345   species were identified in OPR 3: *Desmodesmus* sp. (74%), *T. obliquus* (1%), and others (22%)

346   (Figs. 2 and S4, and Data S3 and S4). Although a greater number of microalgal species were found

347   in OPR 1, the highly dominant microalgal species in both OPRs 1 and 3 was *Desmodesmus* sp.

348   (Fig. 2). The *Desmodesmus* sp. population of OPR 3 was 12% higher than that of OPR 1.

349   As water temperature in OPR 1 and 3 increased in spring (Fig. 1A), some green microalgae

350   (e.g., *Desmodesmus* sp. and *T. obliquus*) showed a change in their taxonomic ranks (Fig. 3). The

351   microalgal bloom period in the spring was ~3–4 weeks because of competition within the

352   cultivation tank, and the effect of increased numbers of other eukaryotic organisms switched every

353   2–3 weeks (Fig. S4). In late spring, green microalgae (Chlorophyta), such as *Desmodesmus* sp.,

354   proliferated rapidly as water temperature increased, becoming the dominant species (Fig. S7A–D).

355   In summer, various species, including green microalgae, such as *Desmodesmus* sp., *Chlorella* sp.,

356   *C. pseudomicroporum*, and *P. integrum*, proliferated more vigorously in OPR 1 compared to OPR

357   3 (Figs. 3, S5A–E, and S8). In addition, the proportion of *Heterocypris* species, known as

358   *Ostracoda*, increased to 33% in OPR 1 in summer (August) before disappearing after the fall when

359   water temperature decreased (Figs. S1A and S9A, and Data S4). In OPR 3, *Nucleariidae* was

360   abundant in summer but disappeared after the fall (Figs. 3 and S9B). In the fall, various species

361   that were highly abundant in summer disappeared because of low water temperatures (Figs. 1A

362   and S9C–D, and S10). *Desmodesmus* sp. became dominant again in OPRs 1 and 3, and the

363   proportions of *Coelastrum* sp., *Golenkinia longispicula*, and *Micractinium* sp. increased in OPR

364   1. *Chlorella miniata* appeared in the fall as well (Figs. 3 and S10A). Overall, our results indicated

365   that the difference between ecologically stabilized (OPR 3) and artificial (OPR 1) cultivation

366   systems can be distinguished by their microalgal flora contents and that *Desmodesmus* sp. is a

367   potential species for mass cultivation, especially in an open pond system.

368

369   *Cyanobacterial community during mass cultivation*

370   We identified 26 cyanobacteria species (Figs. S11 and S12, and Data S5 and S6). Of these,

371   *Cyanobium gracile* frequently appeared in both OPR 1 and 3 in spring and summer, while

372   *Foliisarcina bertiogensis*, *Spirulina major, Cyanobacterium aponinum*, and *Microcystis*

373   *aeruginosa* bloomed in the fall, but their populations were higher in OPR 1 compared to OPR 3,

374   and *Synechococcus elongatus* and *Calothrix desertica* were identified only in OPR 3 in the fall

375 (Fig. S12F–H). The relative abundance of other cyanobacterial species identified was < 1% (Fig.

376 S14). Overall, the number of emerging microalgal species in OPRs 1 and 3 was high in summer

377 and low in spring and the fall, consistent with cyanobacterial species emergence, which are highly

378 abundant in summer. In addition, three dinoflagellate and one cryptomonad species were also

379 identified. In wastewater treatment coupled with biofuel applications, cyanobacteria continue to

380 dominate during the fall and winter, which has an overarching influence on microalgal diversity

381 and microalgal biomass (Cho et al., 2015). Therefore, we need a good understanding of beneficial

382 interactions between microalgae and other organisms, including cyanobacteria, for exploring the

383 biotechnological applications of microalgae-based biomasses.

384

385 **Proximate and ultimate analyses**

386 A higher percentage of bioenergy parameters such as volatile matter (VM; > 80%) is advantageous

387 for biofuel production and biodiesel quality, so we performed quantitative estimation of the

388 microalgal biomass using proximate and ultimate analyses (Awaluddin et al., 2016). We identified

389 very small differences between OPRs 1 and 3 during culture. In proximate results, moisture, VM,

390 and ash contents were 4.2–8.3, 71.5–84.0, and 10.5–21.7 wt%, respectively. In addition, moisture

391 levels were slightly higher in spring and the fall in OPR 1, although it was difficult to determine

392 a regular pattern for moisture in OPRs 1 and 3. The increased moisture value in spring gradually

393 decreased in summer and again increased in the fall, which was observed in both OPRs 1 and 3.

394 The VM content was the highest in summer and the fall, with OPR 1 being ~2%–3% higher than

395 OPR 3, while the ash content reached the highest level in spring, with OPR 3 being ~4%–5%

396 higher than OPR 1. Overall, the mean weight percentage values of moisture, VM, and ash content

397 were 6.68, 79.73, and 13.57, respectively, in OPR 1 and 6.10, 77.42, and 16.48, respectively, in

398 OPR 3 (Table 1).

399 In ultimate, carbon, nitrogen, and hydrogen levels were relatively higher in summer and

400 the fall relative to levels in spring and higher in OPR 1 compared to OPR 3, consistent with the

401 increased water temperature during culture. The sulfur and oxygen content were also higher in

402 OPR 1 compared to OPR 3. The mean weight percentage values of carbon, hydrogen, nitrogen,

403 sulfur, and oxygen (CHNSO) were 43.22, 6.23, 6.23, 0.46, and 30.31, respectively, in OPR 1 and

404 41.50, 5.86, 5.62, 0.41, and 26.77, respectively, in OPR 3. The CV was higher in summer and the

405 fall compared to spring and higher in OPR 1 compared to OPR 3 (Table 1). The mean CV of OPR

406   1 and 3 was 19.51 and 18.43 MJ·kg^{-1}, respectively, which are higher compared to the land plant

407   biomass (18.4 MJ·kg^{-1}) (Ross et al., 2008). Therefore, the microalgal biomass grown in an open

408   pond using mass cultivation, especially OPR 1, can have the potential to be used for producing

409   biofuels of the desired quality.

410

411   **Fatty acid profiles**

412   Figure 4 shows the fatty acid compositions of the microalgal biomasses in OPRs 1 and 3 during

413   mass cultivation from April to November 2017. The major fatty acids analyzed were myristic

414   (C14:0), palmitic (C16:0), palmitoleic (C16:1), palmitidonic (C16:4), oleic (C18:1), linolelaidic

415   (C18:2), and linolenic (C18:3) acids. C16:0 was the most abundant fatty acid in both OPRs

416   throughout the culture period (21.9%–36.5%), while C18:1 was the second-most abundant fatty

417   acid in OPR 1, which increased from spring to summer when water temperature was high and then

418   decreased in the fall. In contrast, C18:1 increased in OPR 3 in spring and the fall and slightly

419   decreased in summer (range 10.1%–28.6%). In addition, the C16:1 ratio reached a maximum of

420   25.6% in both OPRs, showing a similar pattern as C16:0 in OPR 1 and gradually decreasing with

421   time in OPR 3. The staple lipid composition for biodiesel production was better in OPR 3

422   compared to OPR 1, except for C16:1. In addition, we found traces of other fatty acids and no

423   significant difference between OPRs 1 and 3. However, we identified arachidonic acid (C20:4;

424   0.19%) and eicosapentaenoic acid (C20:5; 1.38%) only in OPR 1 and behenic acid (C22:0; 0.19%)

425   in OPR 3. Also, the mean C16:0, C18:1, and C18:2 composition of the biodiesel was 24.5%,

426   17.0%, and 6.1%, respectively, in OPR 1 (47.6% in total) and 24.2%, 22.0%, and 5.8%,

427   respectively, in OPR 3 (52% in total). The total UFA concentration in the biodiesels derived from

428   OPR 1 and 3 was 65.1% and 67.2%, respectively, approximately twice the saturated fatty acid

429   (SFA) concentrations (34.9% and 32.8%). Therefore, the microalgal biomass produced through

430   mass cultivation, especially OPR 3 with a high C18:1 content, has potential as feedstock for

431   producing biodiesel of the desired quality.

432

433   **Biodiesel quality evaluation**

434   The viscosity, ignition quality, oxidative stability, and cold flow of biodiesel are largely affected

435   by the structure of component fatty acid esters (Arguelles et al., 2018). Therefore, it is important

436   to develop a microalgal species with a suitable lipid composition for microalgae-based biodiesel

437 production. In this study, we calculated fuel physicochemical properties using empirical equations

438 (based on fatty acid profile analysis) because proximate and ultimate analyses and fatty acid

439 profiling showed that mass-cultured biomass has the potential to be used in biofuel production

440 (Table 1 and Fig. 4). The parameters analyzed included SV, IV, DU, LCSF, CFPP, oxidation

441 stability, kinematic viscosity, and density. Fuel quality properties were also determined using

442 empirical equations and were within the limits of biodiesel standards ASTM D6751 and EN 14214.

443 The biodiesels derived from OPRs 1 and 3 had a low density (0.87 g·cm^{-3}) and low kinematic

444 viscosity (3.95 mm^2·s^{-1}). In addition, values of the other parameters were as follows: CN, 56.27

445 and 52.97, respectively; oxidation stability, 13.02 and 11.66 h, respectively; CFPP, –3.8°C and –

446 3.0°C, respectively; and IV, 85.83 g·I$_2$·(100 g)$^{-1}$ and 97.16 g·I$_2$·(100 g)$^{-1}$ fat, respectively. The

447 mean SV, DU, and LCSF were 203.60, 62.75 wt%, and 4.03 wt%, respectively, in OPR 1 and

448 201.83, 69.37 wt%, and 4.31 wt%, respectively, in OPR 3 (Table 2).

449

450 **Possibility of microalgae as biological enhancers**

451 The microalgal biomasses in OPRs 1 and 3 had the potential of feedstock for biodiesel production.

452 Microalgae have been used in various industries, such as biofuel, cosmetics, bioremediation, and

453 biocontrol, so we examined the application of microalgae as bioenhancers in soybean plants

454 (Ronga et al., 2019). As shown in Fig. 5A–P, the microalgal population biodiversity in 2018 was

455 similar to that in 2017 during mass culture, with the dominant species being *Desmosdesmus*. Figure

456 5Q shows weather changes during the analyzed periods (July 20 to November 21, 2018). The most

457 distinguishable environmental change was a gradual decrease in air temperature. We conducted

458 environmental adaptation assessment at least twice with V2 stage soybean plants grown after seed

459 germination. The microalgae bioenhancer ability was based on the phenotype and seed

460 germination. The first assessment found that the group subjected to the microalgal biomass (1–4

461 and 1–5), clear solution (1–3), or spray (1–6) of OPR 1 showed improved growth development

462 compared to control groups (1–1 and 1–2), while groups treated with the clear solution (3–3) or

463 spray (3–6) of OPR 3 showed an improved phenotype compared to other groups (Figs. 6A–E and

464 S13). In addition, the soybean plant seeds treated with cleared microalgal culture solutions, which

465 included microalgal biomass (1–4 and 1–5), had better quality compared to other groups, resulting

466 in increased pod seed weight (Figs. 6F–H and S13). Therefore, the microalgal biomasses and

467 supernatants of mass cultures can be used as feedstock for producing biodiesels and biofertilizers,

468   respectively.

469

470   **Discussion**

471   **Overview of the MiSeq-based microalgal community during mass cultivation**

472   In previous studies, MiSeq of the 18S rRNA V9 region has failed to determine some species or

473   their abundance in dinoflagellate-diatom mixtures simulated at different ratios (Guo, Sui & Liu,

474   2016). Therefore, various universal primers against 18S rRNA regions have been used to identify

475   microalgal species at the molecular level. Although actin is more suitable as a molecular marker

476   compared to the 18S rRNA gene for analyzing dinoflagellate communities (Guo, Sui & Liu, 2016),

477   the 18S rRNA gene is more effective for HTS compared to other candidate genes because 400–

478   500 bp amplicons are required (Fig. S1) (Ishaq & Wright, 2014). In addition, various regions of

479   the 18S rRNA gene have been introduced into HTS-based comparative analysis. Of these, the V4

480   and V8–V9 regions are significant, and taxonomic groups for the V8–V9 region exhibit higher

481   concordance rates compared to the V4 region (Fig. S1). In general, microalgal communities are

482   mostly profiled through morphological identification, followed by qualitative/quantitative

483   assessment using DNA barcoding (Serrana et al., 2019). However, this method is not optimal for

484   monitoring microalgal biodiversity in OPRs because of interfering factors, such as the cultivation

485   cycle (month, season, and year) (Cheung, Allen & Short, 2020). Metagenomic analysis based on

486   DNA barcoding (the V8–V9 region of the 18S rRNA gene) can be used for rapid, accurate, and

487   automatable identification of microalgal species and their precise abundances in microalgal

488   cultures of industrial interest.

489

490   **Changes in nutrient parameters during mass cultivation in open pond raceways**

491   With regard to weather changes and nutrient (e.g., nitrogen and phosphorus) consumption, the

492   temperature of OPR 1 was higher compared to OPR 3 during the mass cultivation period except

493   in summer. pH was neutral to heavy alkaline (7.2–10.4) and became increasingly neutral with a

494   gradual increase in water temperature (Fig. 1B). TN and TP consumption was 12.87 mg·L^{-1}

495   (86.2%) and 2.26 mg·L^{-1} (96.3%), respectively, in OPR 1 and 13.14 mg·L^{-1} (90.1%) and 2.10

496   mg·L^{-1} (93.5%), respectively, in OPR 3 (Fig. 1C), confirming a higher nutrient removal rate for

497   TP compared to TN. Phosphorus plays an important role in microalgae cellular metabolism by

498   forming many structural and functional components required for growth and development (Znad

499 et al., 2018). Naturally occurring mixed microalgae have been used for wastewater treatment under
500 OPR conditions. Posadas et al. (2015) showed in OPR cultivation of *Scenedesmus* sp. that
501 wastewater treatment is confirmed by the effect of the CO_2 source. The TN and TP removal rates
502 were 74% ± 14 and 57% ± 12, respectively. In another study involving semi-continuous cultivation
503 of *Chlorella* sp., *Scenedesmus* sp., and *Stigeoclonium* sp., wastewater treatment showed a mean
504 TN and TP removal rate of 76.3% ± 4.0 and 51.5% ± 3.9, respectively (Kim et al., 2018).
505

506 **Algal biomass productivity**
507 The DW of OPR 3 was higher compared to OPR 1 during June–August 2017, with the highest
508 difference in summer, when water temperature increased. OPR 3 had a maximum biomass
509 production of 17.6 g·m^{-2}·d^{-1}, while OPR 1 in the greenhouse had a maximum biomass of 13.3
510 g·m^{-2}·d^{-1} (Fig. 1E). Under specific environmental conditions of Almería, *Scenedesmus* is
511 consistently the dominant microalgal species in OPRs. In previous studies, biomass productivities
512 ranged from 4 ± 0 g·m^{-2}·d^{-1} in December to 17 ± 1 g·m^{-2}·d^{-1} in July (Posadas et al., 2015). The
513 two OPRs had a mean total biomass production of ~8.6–9.9 g·m^{-2}·d^{-1}, corresponding to 0.307–
514 0.309 DW·L^{-1} (Fig. 1D–1F). The lower biomass production in OPR 1 was presumably because of
515 the reduced light intensity caused by the greenhouse's semitransparent film, as previously reported
516 (Giacomelli & Roberts, 1993). Supplying *Desmodesmus* sp. F51 with inorganic carbon and
517 nitrogen sources at a 1:1 ratio enhanced cell growth and improved biomass production up to 939
518 mg·L^{-1}·d^{-1} (Xie et al., 2017). Overall, biomass and DW values corresponded to nutrient
519 consumption, highlighting the time when the water temperature was maintained at 20 °C–25 °C,
520 on average, because of the higher atmospheric temperatures from June to September. Microalgae
521 had an optimum growth temperature of 20 °C–25 °C under normal conditions (Patel et al., 2019).
522

523 **Microalgal and cyanobacterial communities**
524 Although we found a large number of microalgal species, including *Desmodesmus* sp., *Chlorella*
525 sp., *T. obliquus*, and *P. integrum*, in both OPRs 1 and 3 in a 9 month period, the microalgal species
526 highly dominant in both OPRs was *Desmodesmus* sp.. The microalgal abundance of OPR 1 was
527 greater compared to OPR 3. Seasonal changes in light conditions play an important role in
528 sustaining species diversity in OPR systems. The richness of *Desmodesmus* sp. in OPR 3 was 12%
529 higher compared to OPR 1 (Fig. 2). Similarly, in the case of two full-scale wastewater high rate

530   algal ponds (HRAPs) (NHRAPs and SHRAPs) operated over a 23 month cultivation period from

531   spring 2015 to winter 2016 in New Zealand, *Desmodesmus* sp., *Coelastrum* sp., *Micractinium* sp.,

532   *Microcystis* sp., and *Pediastrum* sp. were the wastewater microalgal community representative of

533   the dominant species, which changed either seasonally (e.g., with retention time and loading rates)

534   or with species manipulation conditions (e.g., microalgal recycling) (Sutherland, Turnbull &

535   Craggs, 2017; Lutzu & Dunford, 2018). *Micractinium pusillum* dominated the microalgal

536   community for an 18 month cultivation period in the two HRAP systems (Sutherland, Turnbull &

537   Craggs, 2017), while *Desmodesmus* sp. dominated the microalgal community for a 9 month

538   cultivation period despite changes in multiple environmental variables in the two OPR systems in

539   our study. Cho et al. (2015) performed mass cultivation with untreated municipal wastewater in

540   an HRAP for 1 year (2013–2014) in Korea. Observation of the microalgal diversity in semi-

541   continuous mode showed that the dominant microalgal genera in all four seasons were

542   *Scenedesmus*, *Microcystis*, and *Chlorella*. In contrast to our results, *Scenedesmus* sp. persisted in

543   Cho et al.'s (2015) culture system in all four seasons, but the three strains were not co-dominant..

544   To identify the most promising species that can be used as biodiesel feedstock for a large-scale

545   cultivation system in China. Xia et al. (2014) cultivated *Desmodesmus* sp. and *Scenedesmus* sp.

546   under outdoor conditions. In waste water treatment coupled with an HRAP, the *Scenedesmus* sp.

547   population, which was the dominant strain in summer (initial stages of cultivation), decreased

548   significantly and *Microcystis* sp. became the dominant species, although it was absent in the

549   preceding months. In spring and summer, *Chlorella* was the dominant genus (Cho et al., 2015).

550   The change in dominance from one genus to another was drastic and rapid, typically within 7–10

551   days (Fig. 2). The turnover rate of dominant species in natural systems may be equally fast when

552   the steady-state phase beaks down because of significant shifts in environmental changes, although

553   the low sampling frequency of many studies has made this difficult to quantify (Sutherland,

554   Turnbull & Craggs, 2017). In the HRAP-dependent wastewater treatment process, dynamics of the

555   *Scenedesmus* population are highly correlated with temperature, while those of the *Chlorella*

556   population are highly correlated with the effluent's organic content (Cho et al., 2015). Therefore,

557   monitoring the rapid turnover of the dominant species, as shown in our study, might make it

558   challenging to maintain a desirable species at the full-scale.

559   Microalgal diversity and biomass are likely affected by different parameters, such as

560   environmental factors (e.g., temperature) and to biological and physiochemical parameters (e.g.,

561   influent microbial diversity and organic carbon, nitrates, pH, dissolved oxygen, and conductivity

562   levels) (Zongo et al., 2019). However, the most significant factor affecting species abundance is

563   temperature. Variations in the local environment (i.e., in the temperature of the immediate

564   surroundings of a community) are crucial, not only for the ecosystem process rate, but also for

565   species assembly persistence and the direct relationship between biodiversity and ecosystem

566   functioning.

567   In the OPRs 1 and 3 systems, we found no differences in biomass productivity (mean: 8.6–

568   9.9 g·m^{-2}·d^{-1}) and nutrient consumption rates of TN and TP (Figs. 1C and 1F). Accordingly, in

569   microalgal communities in the two full-scale wastewater HRAPs, ~33 microalgal species were

570   found but species abundance was low and not related to either productivity or nutrient removal

571   efficiency (Sutherland, Turnbull & Craggs, 2017). As shown in our study, both nutrient removal

572   and biomass production did not differ between the two HRAPs when the dominant species was

573   the same or different between the two ponds (Sutherland, Turnbull & Craggs, 2017). These

574   findings indicate that compared to individual species, microalgal communities are more critical

575   for OPR performance. In addition, microalgae mass cultivation for the producing biomass and

576   associated valuable compounds have gained increasing interest not only within the scientific

577   community but also at the industrial level.

578   In our study, we identified ~26 cyanobacteria species (Figs. S11 and S12), of which

579   *Cyanobium gracile* frequently appeared in both OPRs 1 and 3 in spring and summer, while

580   *Foliisarcina bertiogensis*, *Spirulina major*, *Cyanobacterium aponinum*, and *Microcystis*

581   *aeruginosa* bloomed in the fall. However, their populations were higher in OPR 1 compared to

582   OPR 3. *Synechococcus elongatus* and *Calothrix desertica* were identified only in OPR 3 in the fall

583   (Fig. S12). In the two full-scale wastewater HRAPs, identified cyanobacteria, such as

584   *Dolichospermum circinale*, *Merismopedia tenuissima*, *M. aeruginosa*, *Microcystis flosaquae*,

585   *Oscillatoria* sp., and *Planktothrix isothrix*, with the dominant species being *M. aeruginosa*

586   (Sutherland, Turnbull & Craggs, 2017). The high richness of *M. aeruginosa* for a short duration

587   resulted in a change in the dominant species (Sutherland, Turnbull & Craggs, 2017). In addition

588   to the microalgal community, nutrient concentrations of nitrogen and phosphorus and organic

589   carbon loads affect cyanobacteria dominance (Cho et al., 2015).

590   Under harsh conditions, *Desmodesmus* sp. produces a higher amount starch grains, oil bodies,

591   and cell wall polysaccharides and increases its symbiotic interactions with taxonomically distant

592   invertebrates (e.g., hydroids, sponges, and polychaetes) (Baulina et al., 2016). These symbiotic

593   relationships include all possible interactions known in nature, such as mutualism, commensalism,

594   and parasitism (McKay, Gibbs, & Vaughn, 1991). Cyanobacteria can also have symbiotic

595   interactions with microalgae. For example, the co-culture of the Louisiana-native microalga

596   *Chlorella vulgaris* and the cyanobacterium *Leptolyngbya* sp. shows commensalism to produce

597   lipids for biodiesel production (Subashchandrabose et al., 2011). On the basic of the symbiotic

598   relationship, microalgae cyanobacteria consortia can lead to a high microalgal growth rate,

599   improved nutrient and pollutant uptake, and production of metabolites with high biotechnological

600   application potential (Silaban et al., 2014). The exact metabolic mechanisms underlying such

601   interactions are unclear. However, the benefits of this growth strategy can potentially be exploited

602   in different biotechnology fields, such as biofuel production, especially if the microalgal species

603   involved possesses a high lipid contents (Lutzu & Dunford, 2018).

604

605   **Characteristics of microalgal biomass, and fatty acids in mass cultivation**

606   The microalgal community is a more important determinant of freshwater systems and microalgal

607   biomass production in OPRs 1 and 3 compared to one individual species. The mean weight

608   percentage CHNSO values were 43.22, 6.23, 6.23, 0.46, and 30.31, respectively, in OPR 1 and

609   41.50, 5.86, 5.62, 0.41, and 26.77, respectively, in OPR 3. The mean weight percentage CHNSO

610   values of *Hapalosiphon* sp. biomass are 47.94, 7.44, 6.45, 0.58, and 37.58, respectively, while

611   those of *Botryococcus braunii* biomass are 77.04, 12.40, 1.23, 0.18, and 9.86, respectively (Liu et

612   al., 2012). In our study, the CVs were higher in summer and the fall compared to spring and were

613   higher in OPR 1 compared to OPR 3 (Table 1). The mean CVs of OPR 1 and 3 were 19.5 and 18.5

614   MJ·kg^{-1}, respectively, which are higher compared to the land plant biomass (18.4 MJ·kg^{-1}) (Ross

615   et al., 2008). In general, microalgae grown under normal conditions have a CV of 18–21 MJ·kg^{-1}

616   (Scragg et al., 2002). The CV of *Hapalosiphon* sp. is 14.75 MJ·kg^{-1}, similar to that of sewage

617   sludge or lignocellulose biomass (19–25 MJ·kg^{-1}), while the CV of *B. braunii* is as high as 35.58

618   MJ·kg^{-1} because of total carbon and hydrogen contents of 89.4% in the microalgal biomass, similar

619   to that of crude oil (Liu et al., 2012). Considering a high carbon and hydrogen content an advantage

620   for feedback in biofuel production, our results showed that the microalgal biomasses in OPRs 1

621   and 3 were feasible for biodiesel production, although the produced values were lower compared

622   to *B. braunii*.

623   The major fatty acids of the microalgal biomasses in OPRs 1 and 3 were C16:0 (24.5% and 24.2%,

624   respectively), C16:1 (15.4% and 9.7%, respectively), C16:4 (6.0% and 7.6%, respectively), C18:1

625   (16.9% and 21.9%, respectively), C18:2 (6.1% and 5.8%, respectively), and C18:3 (14.1% and

626   17.9%, respectively) (Fig. 4). The fatty acids produced by microalgae are C16:0, C18:0, C18:1,

627   C18:2, and C18:3 (Huang et al., 2015), of which UFAs (C18:1 and C18:2) are important

628   components of high-quality biodiesel (Bouaid, Martinez & Aracil, 2007). In our study, mean

629   C16:0, C18:1, and C18:3 content of the biodiesel was 24.5%, 17.0%, and 14.5%, respectively, in

630   OPR 1 (56.0% in total) and 24.2%, 22.0%, and 17.9%, respectively, in OPR 3 (64.1% in total)

631   (Fig. 4). Especially, C18:1 is an important indicator of biodiesel quality and provides great stability

632   (Ho et al., 2014). The total UFA concentration in the biodiesels derived from OPRs 1 and 3 was

633   65.1% and 67.2%, respectively, which was approximately two times greater compared to SFAs

634   (34.9% and 32.8%, respectively). The low PUFA content and high SFA and MUFA content are

635   among the most important indicators of biodiesel quality (Piligaev et al., 2018). The fatty acid

636   profiles of microalgal oils generally affect the qualities of the biodiesel produced. High SFA and

637   MUFA contents lead to high oxidative and thermal stabilities, resulting in a slower deterioration

638   rate of lipid characteristics (Mostafa & El-Gendy, 2017).

639   In our study, we found high levels of fatty acids with a high degree of SFAs, such as C16:0.

640   In addition, MUFAs C16:1 and C18:1 were abundant in OPRs 1 and 3 (Fig. 4). According to

641   biodiesel standard EN 14214, the percentages of linolenic acid (C18:3) and PUFAs (P4 double

642   bond) should not be greater than 12% and 1%, respectively (Mandotra et al., 2014). The fatty acid

643   profiles of microalgal biomasses in OPRs 1 and 3 had a high linolenic acid content of 14.2% and

644   18.0%, respectively. Assessment of the amount of FAMEs with $\geq$ 4 double bonds for microalgal

645   crude oil extract has shown that the microalgal strain does not produce, FAMEs and therefore

646   represents a good biodiesel source (Mandotra et al., 2014). Microalgal fatty acid profiling of

647   biodiesel obtained from *Desmodesmus* sp. I-AU1 contains a total SFA methyl ester content of

648   31.0%, while the total MUFA C18:1 content is 25.6% (Arguelles et al., 2018). The SFA and

649   MUFA percentage composition of the microalga was 56.7%, which is high relative to related

650   studies (Arguelles et al., 2018). The fatty acid content of microalgal biomasses in OPRs 1 and 3

651   mainly comprised SFAs and MUFAs (67.3% and 64.8%, respectively), which are suitable for

652   biodiesel production.

653

654 **Properties of microalgal biomass-based biodiesel quality parameters in mass cultivation**

655 The fatty acid profile of microalgal oils generally affects biodiesel quality (Nascimento et al.,

656 2013). The biodiesels derived from OPRs 1 and 3 had a low density (0.87 g·cm^{-3}) and low

657 kinematic viscosity (3.95 mm^2·s^{-1}). In addition, values of the other parameters were as follows:

658 CN, 56.27 and 52.97, respectively; oxidation stability, 13.02 and 11.66 h, respectively; CFPP, −

659 3.8°C and −3.0°C, respectively; and IV, 85.83 g I$_2$·(100 g)$^{-1}$ fat and 97.16 g I$_2$·(100 g)$^{-1}$ fat,

660 respectively. The mean SV, DU, and LCSF were 203.60, 62.75 wt%, and 4.03 wt%, respectively,

661 in OPR 1 and 201.83, 69.37 wt%, and 4.31 wt%, respectively, in OPR 3 (Table 2). The carbon

662 chain lengths of both SFAs and UFAs in crude oil extracts generally affect biodiesel properties,

663 such as CN, oxidative stability, and cold–flow properties (Samorì et al., 2013). Generally, crude

664 oil extracts with a high proportion of SFAs and MUFAs are preferred for biodiesel production

665 because these fatty acids increase the energy yield and oxidative stability of biodiesel. On the one

666 hand, oils containing MUFAs are prone to solidification at low temperature; on the other hand,

667 oils rich in PUFAs have good cold-flow properties but are vulnerable to oxidation. This has an

668 adverse effect on fuel conservation and combustion (Arguelles et al., 2018). The total mean

669 percentages of SFAs and MUFAs and of SFAs and PUFAs for the total biomass were 67.3% and

670 64.7%, respectively, and 67.6% and 68.4%, respectively (Fig. 4). With regard to biodiesel quality,

671 microalgal biomasses in OPRs 1 and 3 showed good fuel viscosity, equal or lower density, higher

672 CN and oxidation stability, and good performances at low temperature. Overall, our results

673 indicate that biodiesel produced from the oil *Desmodesmus* sp. grown in open ponds (OPR 1 or 3)

674 will have low emissions, good combustion, good flow properties at low temperatures, and

675 excellent oxidation stability.

676

677 **Application of microalgae as biological enhancers**

678 The bioenhancing ability of microalgae is based on the phenotype. The first assessment showed

679 that the group subjected to the microalgal biomass (1–4 and 1–5), cleared solution (1–3), or spray

680 (1–6) of OPR 1 has improved growth compared to control groups (1–1 and 1–2), while groups

681 treated with the cleared solution (3–3) or spray (3–6) of OPR 3 display an improved phenotype

682 compared to other groups (Figs. 6A–E and S13). Consistent with our results, microalgae have been

683 considered a potential biofertilizer to replace mainstream synthetic fertilizers in order to enhance

684 the biological and chemical properties of soil, positively affecting plant characteristics and

685   increasing yields (Ronga et al., 2019). For example, inoculation of *C. vulgaris* and *S. dimorphus*

686   increases rice yields by improving nitrogen fixation (Garcia-Gonzalez & Sommerfeld, 2016). The

687   culture solution, cellular extracts, and dry biomass of *Acutodesmus dimorphus* enhances seed

688   germination, plant growth, and floral production in Roma tomato (Jochum, Moncayo & Jo, 2018).

689   Therefore, the microalgal biomass and culture solutions of OPRs 1 and 3 improved the agronomic

690   traits of soybean plants and can be introduced as biological enhancers, such as soybean spray and

691   biofertilizer.

692

Conclusions

694   This study investigated the species diversity of the microalgal community during mass cultivation.

695   *Desmodesmus* sp. was found to be the dominant microalgal species throughout the evaluation

696   period. The microalgal biomasses produced from OPRs 1 and 3 are rich in SFAs (C16:0), MUFAs

697   (C16:1 and C18:1), and PUFAs (C16:4 and C18:3). The biodiesel derived from the microalgal

698   biomasses showed good fuel viscosity, equal or lower density, higher CN and oxidation stability,

699   and good performance at low temperature, meeting the major biodiesel standards. The microalgal

700   biomass and culture solution of OPRs 1 or 3 improved the environmental adaptation of soybean

701   plants. Therefore, OPR-grown *Desmodesmus* sp. is a potential alternative feedstock for producing

702   excellent-quality biodiesel and biofertilizer. In addition, the understanding of microalgal diversity

703   can help sustain a dominant genus in OPR systems, maximizing overall efficiency. Following a

704   decrease in water temperature, eukaryotic diversity is highest in summer compared to spring and

705   the fall. In addition, quantitative analysis of the phytoplankton community, which is important for

706   ecological studies, has problems of low accuracy of species identification (which heavily depends

707   on personnel experience) and low efficiency and is also time consuming. As an alternative to

708   overcome such limitations, our results could be applied to develop an improved technique for

709   accurate and fast quantification of microalgal samples via HTS of barcoding genes.

710

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715

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Figure 1

Nutrient consumption, biomass levels, and lipid content in relation to cultivation parameters from April to November, 2017.

Environmental cultivation factors were monitored by measuring the (A) water temperature and (B) pH. Black circle, OPR 1; white circle, OPR 3. (C) Nutrient consumption rate assessed by examining removal of TN and TP. Red, OPR 1 TN; light-blue, OPR 3 TN; light-red, OPR 1 TP; blue, OPR 3 TP. (D) Biomass monitored by measuring the OD at 680 nm for the indicated time. Light-red circle, OPR 1; light-blue circle, OPR 3. (E, F) Biomass productivity also represented DW (g·L^{-1} and g DW m^{-2}·day^{-1}). Light-red bar, OPR 1; light-blue bar, OPR 3. OPR, open pond raceway; TN, total nitrate; TP, total phosphorous; OD, optical density; DW, dry weight.

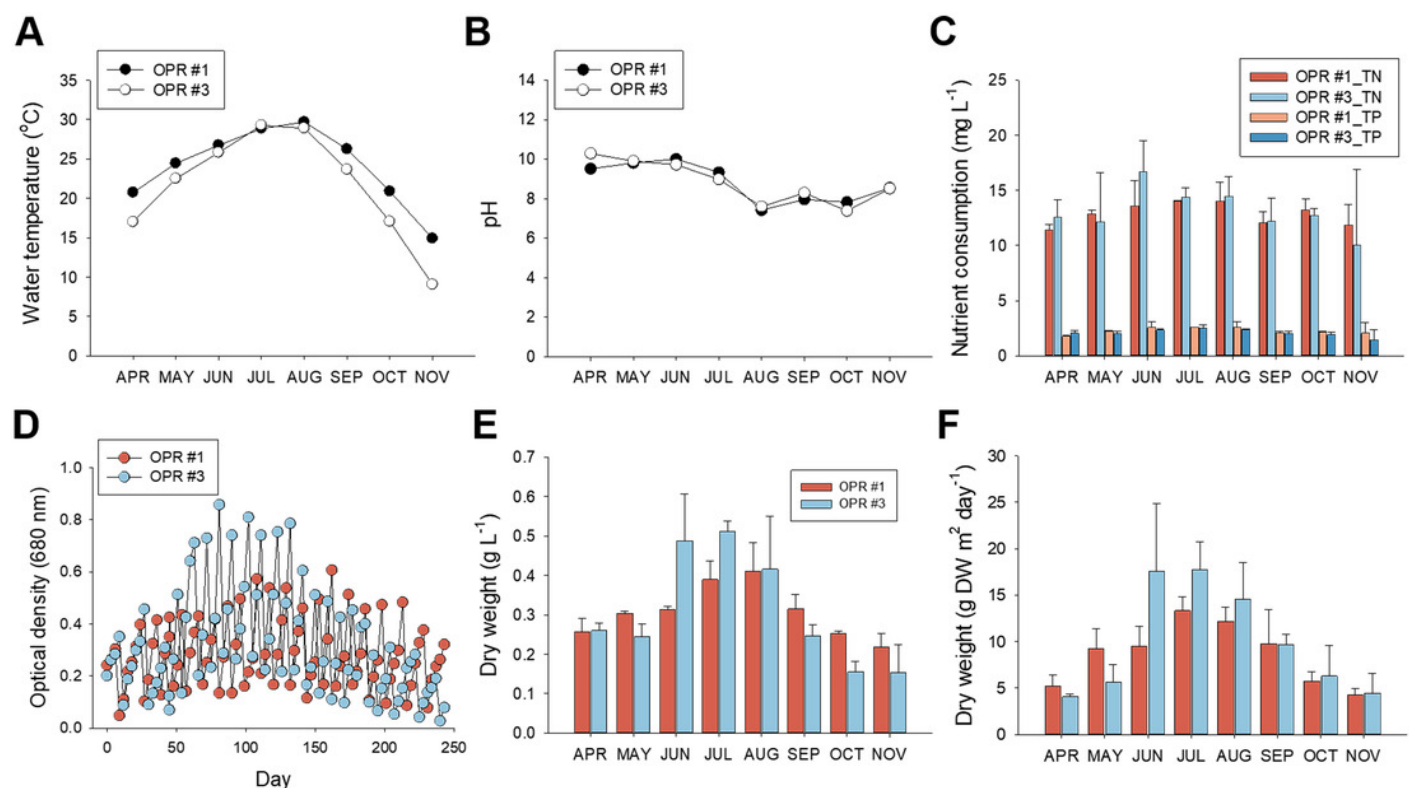



Figure 2

Fig. 2. MiSeq-based identification of the eukaryotic community, including microalgae, from April to November during mass cultivation in OPR 1 and 3.

MiSeq-based identification of the eukaryotic community, including microalgae, from April to November during mass cultivation in (A) OPR 1 and (B) OPR 3. (C) The eukaryotic community was described at the species-level, with ~80 species found in both OPRs 1 and 3, 24 in OPR 1, and 23 in OPR 3. OPR, open pond raceway.

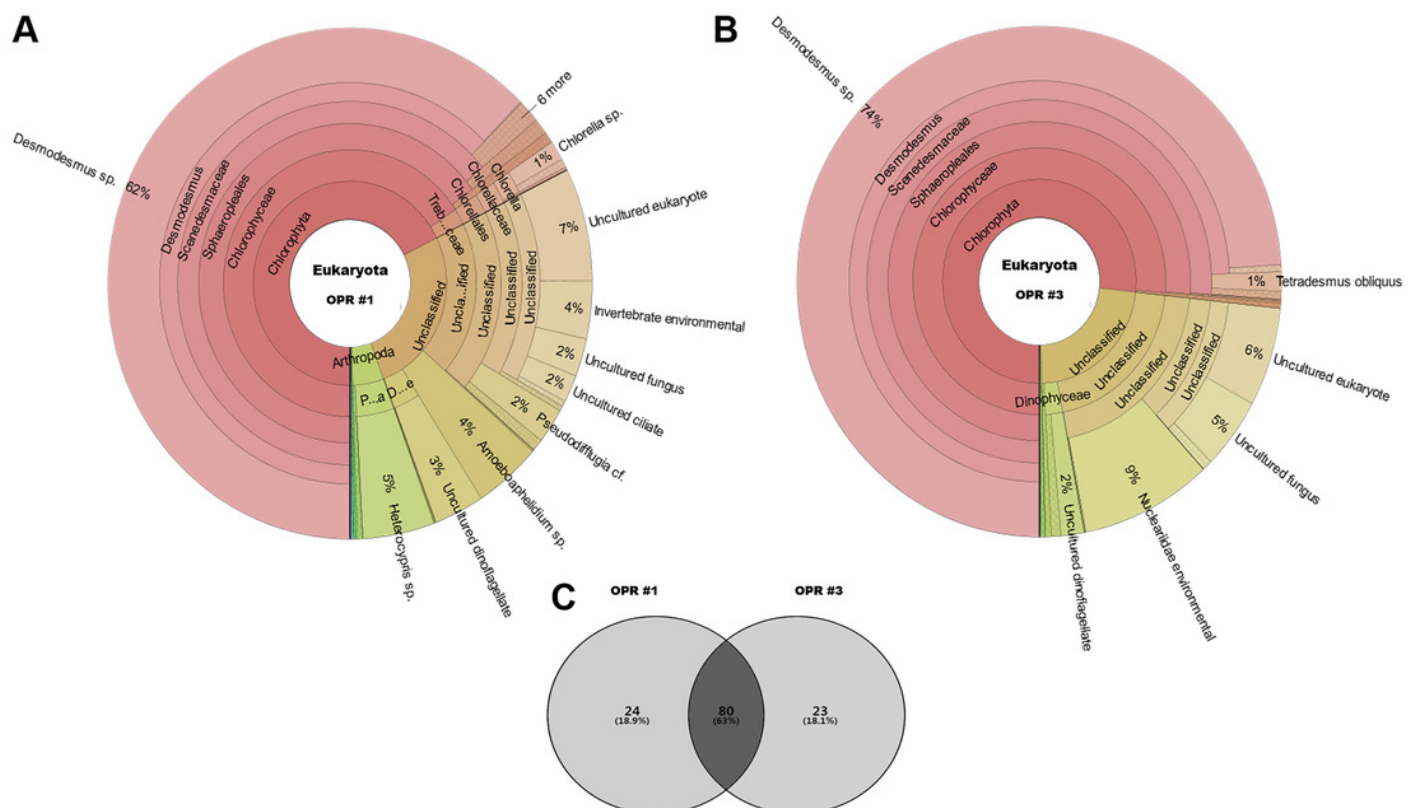



Figure 3

Fig. 3. Microalgal community from April to November during the mass cultivation period. Population abundance of OPR 1 and OPR 3 algal species.

Microalgal community from April to November 2017 during mass cultivation. Population abundance of microalgal species in (A) OPR 1 and (B) OPR 3. Wine, *Desmodesmus* sp.; orange, *Pseudopediastrum integrum*; blue, *Tetradesmus obliquus*; dark cyan, *Chlorella* sp.; magenta, *Chlorella miniata*; dark yellow, others. Microalgal calendar in OPRs 1 and 3 during mass cultivation. Dominant microalgal strain in (C –G) OPR 1 and (P–T) OPR 3 from April to November 2017. Microscopy-based microalgal richness in (H–O) OPR 1 and (U–BB) OPR 3 during the same period. OPR, open pond raceway .

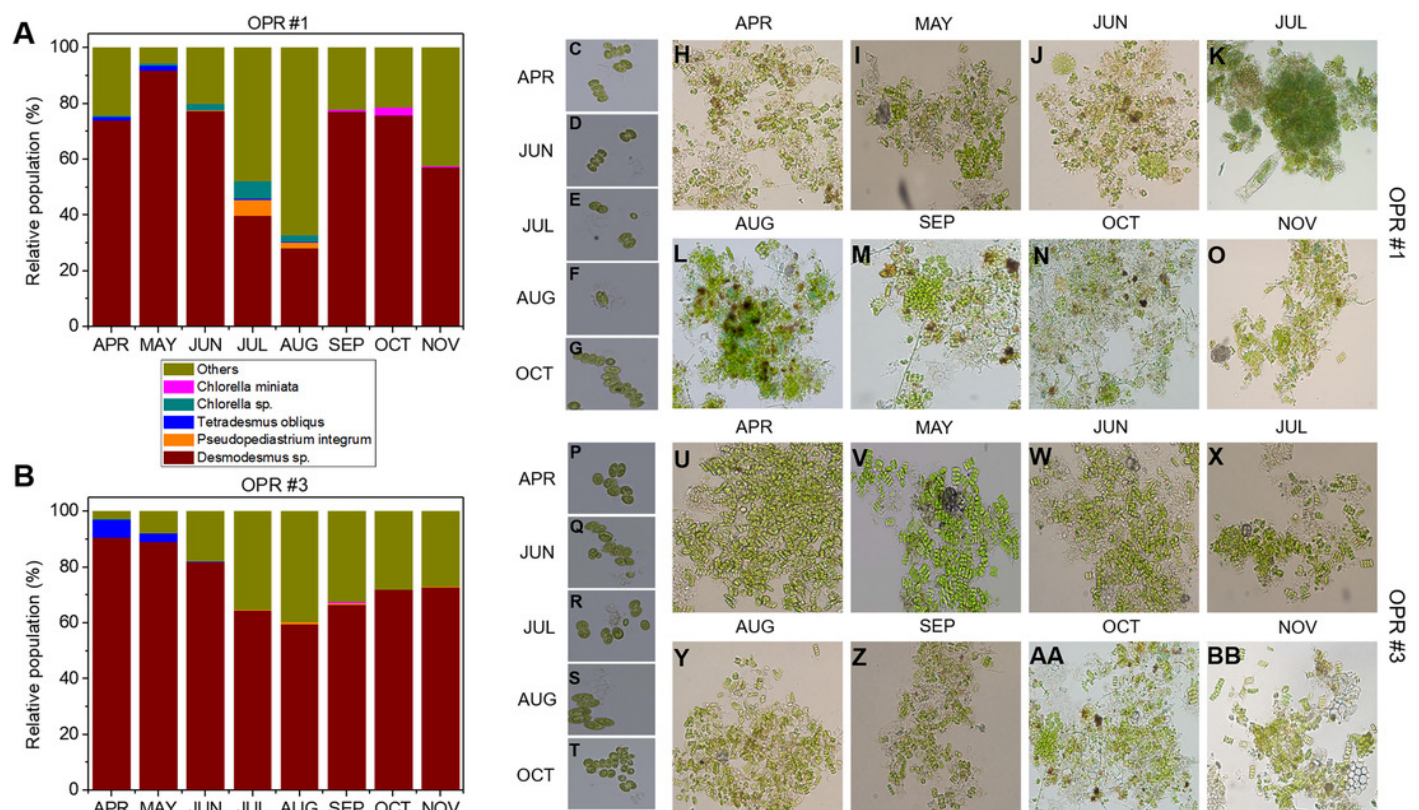



Figure 4

Fig. 4. Relative fatty acid analysis in OPR 1 and OPR 3.

Relative fatty acid analysis in (A) OPR 1 and (B) OPR 3. Fatty acid composition was set at 100%. Wine, C14:0; red, C15:0; light-red, C16:0; apricot, C16:1; incarnadine, C16:2; white, C16:3; light sky blue, C16:4; sky blue, C18:0; light-blue, C18:1; blue, C18:2; navy, C18:3. OPR, open pond raceway.

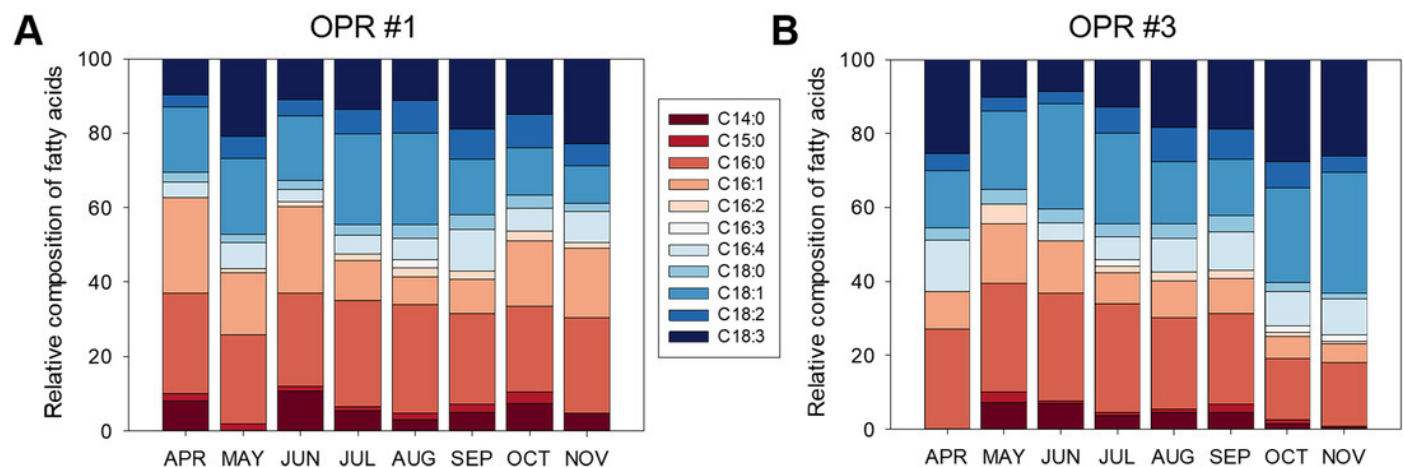



Figure 5

Fig. 5. Microalgal calendar and weather changes during mass cultivation in 2018.

Microalgal calendar and weather changes during mass cultivation in 2018. The dominant microalgal species was similar in 2017 and 2018; the presented images were taken under a microscope at 400× magnification. Microalgal calendar of (A–H) OPR 1 and (I–P) OPR 3 from April to November 2017. (Q) Changes in the mean (square), maximum (circle), and minimum (triangle) temperatures (°C), total sunshine hours (h; star), and precipitation (mm; diamond). OPR, open pond raceway.

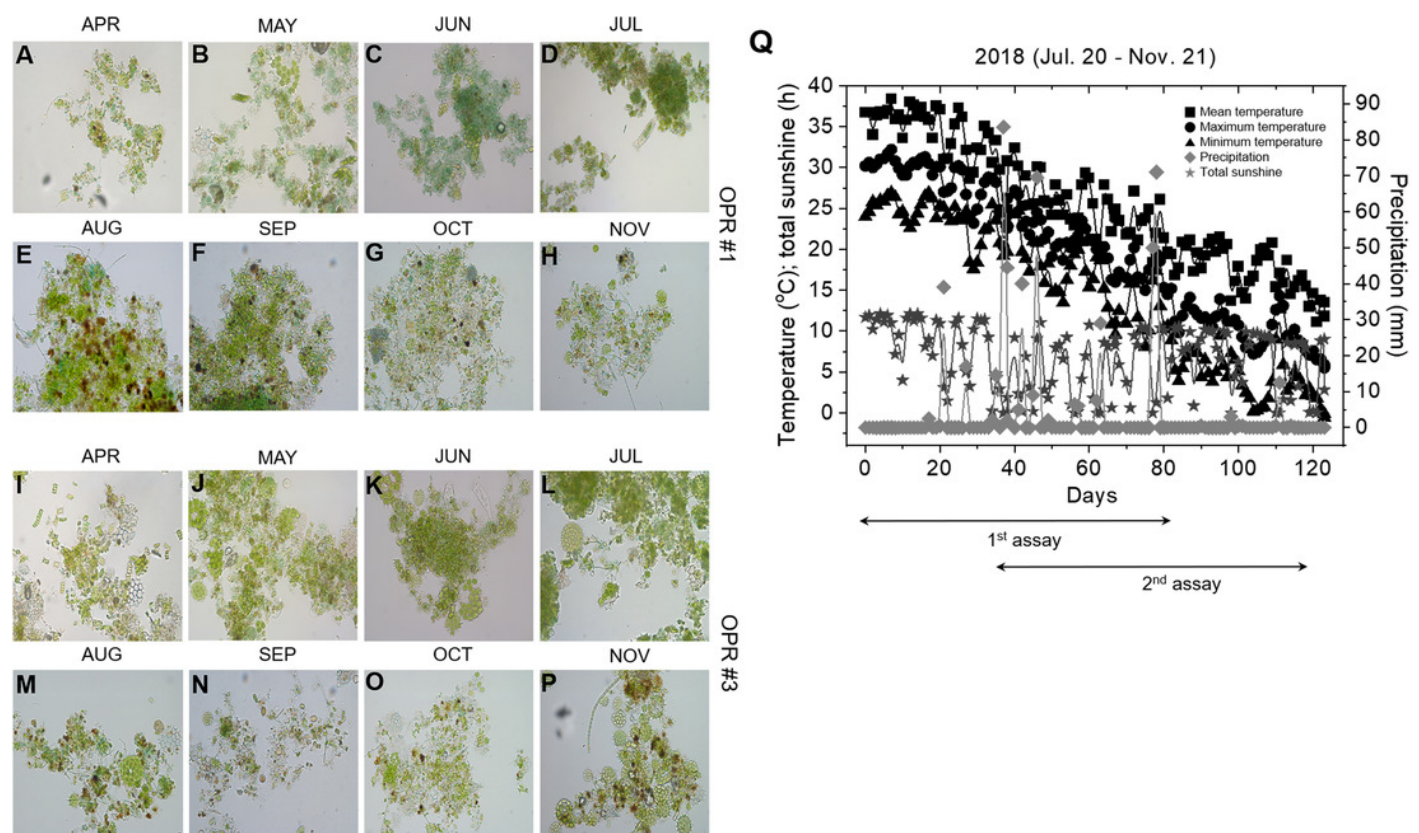



Figure 6

Fig. 6. Biofertilizer assays for soybean plants.

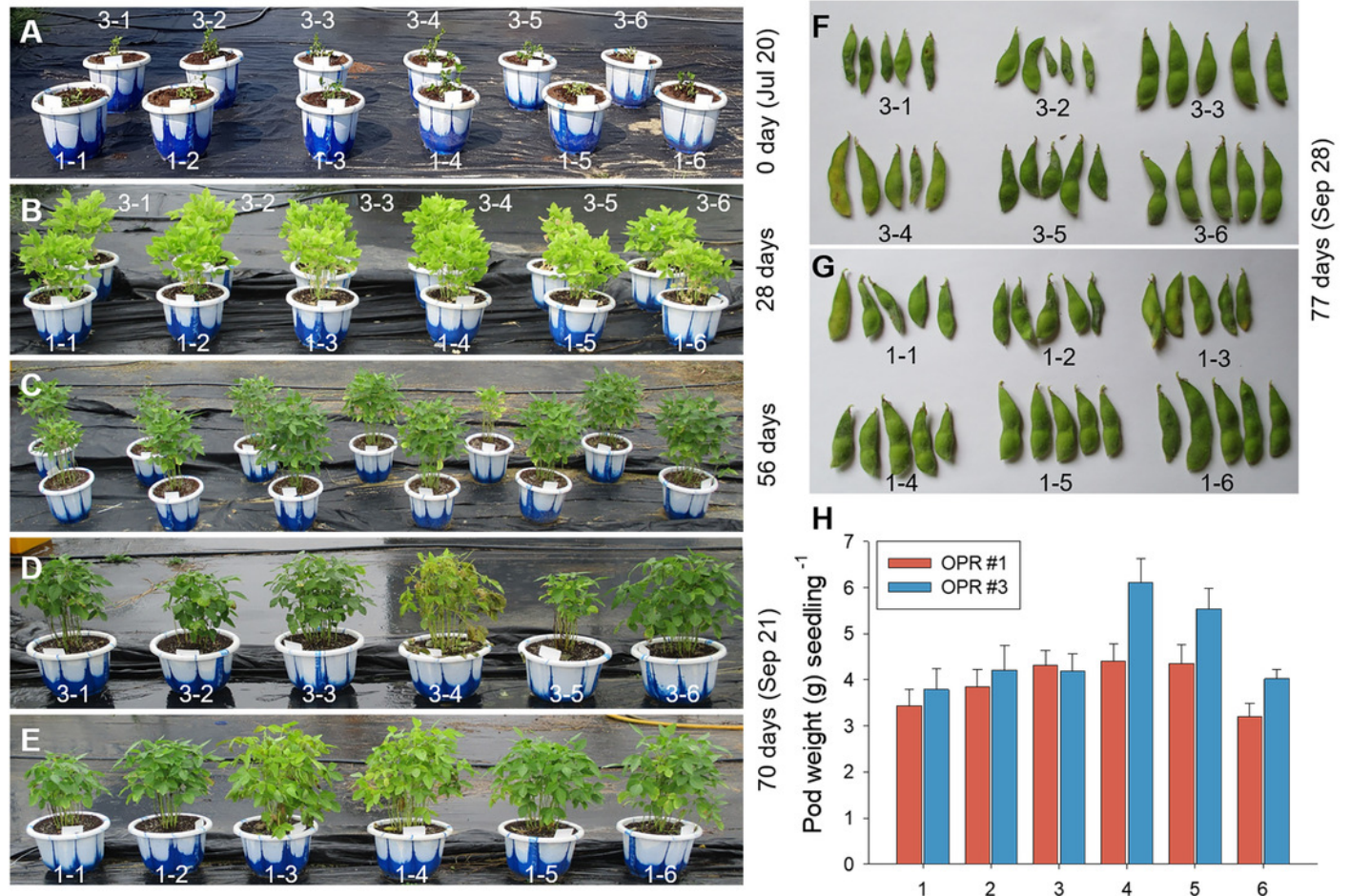
Biofertilizer assays for soybean plants. The first biofertilizer experiment was conducted under field conditions from July 20 to September 28, 2018. Soybean seeds were grown in potting soil. V2 seedlings were transplanted after ~15 days to 30 cm pots (15 seedlings/pot). The plants were grown for 11 weeks and hand-watered, as needed. Phenotypes in the growth stage were represented at (A) 0, (B) 28, (C) 56, and (D, E) 70 days after soybean seedling transplantation. The effects of a mass culture solution as a soil drench were also examined by (F, G) monitoring seed development and (H) weighing the pods per seedling. a, OPR 1; b, OPR 3; 1–1 and 3–1, soybean plants supplemented with groundwater; 1–2 and 3–2, soybean plants supplemented with eco-sol medium; 1–3 and 3–3, soybean plants supplemented with the clear supernatant from OPRs 1 and 3; 1–4 and 3–4, soybean plants supplemented with microalgal biomasses from OPRs 1 and 3; 1–5 and 3–5, soybean plants supplemented with the cultured solution (supernatant plus microalgal biomasses) from OPRs 1 and 3; 1–6 and 3–6, soybean plants sprayed with the clear supernatant from OPRs 1 and 3; gray bar, OPR 1; black bar, OPR 3. OPR, open pond raceway.

Proximate and ultimate analyses of the harvested biomass during mass cultivation

Table 1: Proximate and ultimate analyses of the harvested biomass during mass cultivation

1   Table 1:

	APR		MAY		JUN		JUL		AUG		SEP		OCT		NOV	
	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3
Proximate analysis (wt %)																
Moisture	7.4 ± 0.4	6.3 ± 0.0	8.3 ± 0.2	6.9 ± 0.4	6.3 ± 0.2	7.0 ± 0.1	5.9 ± 0.1	4.2 ± 0.3	5.0 ± 0.0	6.9 ± 0.0	5.2 ± 0.2	6.8 ± 0.2	8.3 ± 0.2	6.2 ± 0.2	7.1 ± 0.1	4.5 ± 0.1
Volatile matter	75.6 ± 0.6	73.6 ± 0.4	76.1 ± 0.2	71.5 ± 0.1	80.3 ± 0.3	76.8 ± 0.6	77.8 ± 0.3	78.5 ± 0.4	84.0 ± 0.1	80.3 ± 0.1	83.2 ± 0.1	81.2 ± 0.4	81.2 ± 0.0	80.8 ± 0.1	79.7 ± 0.1	76.7 ± 0.1
Ash	17.0 ± 0.2	20.1 ± 0.4	15.6 ± 0.4	21.7 ± 0.5	13.4 ± 0.6	16.3 ± 0.5	16.2 ± 0.2	17.3 ± 0.2	11.0 ± 0.1	12.8 ± 0.1	11.7 ± 0.3	12.0 ± 0.2	10.5 ± 0.2	12.9 ± 0.1	13.2 ± 0.3	18.8 ± 0.1
Ultimate analysis (wt %)																
Carbon (C)	39.9 ± 0.4	38.6 ± 0.4	41.7 ± 0.0	39.1 ± 0.7	43.6 ± 0.2	40.8 ± 0.2	42.5 ± 0.1	41.8 ± 0.8	45.8 ± 0.6	45.7 ± 0.2	42.7 ± 0.2	43.0 ± 0.2	46.1 ± 0.0	45.1 ± 0.2	43.5 ± 0.2	37.9 ± 0.5
Hydrogen (H)	5.4 ± 0.1	5.4 ± 0.1	6.0 ± 0.0	5.3 ± 0.1	6.3 ± 0.1	5.7 ± 0.0	6.2 ± 0.0	5.9 ± 0.1	6.6 ± 0.1	6.4 ± 0.0	6.5 ± 0.0	6.5 ± 0.1	6.5 ± 0.1	6.4 ± 0.1	6.4 ± 0.1	5.3 ± 0.1
Nitrogen (N)	5.4 ± 0.1	5.8 ± 0.2	6.2 ± 0.1	5.5 ± 0.2	7.0 ± 0.0	5.4 ± 0.2	5.7 ± 0.1	5.8 ± 0.1	5.3 ± 0.1	6.9 ± 0.0	6.0 ± 0.0	6.0 ± 0.0	7.2 ± 0.1	5.6 ± 0.1	7.1 ± 0.0	4.0 ± 0.1
Sulfur (S)	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
Oxygen (O) (by difference)	31.8 ± 0.4	29.5 ± 0.2	30.2 ± 0.3	28.0 ± 1.5	29.3 ± 0.4	31.6 ± 0.1	28.9 ± 0.2	28.8 ± 1.1	30.9 ± 0.9	27.7 ± 0.0	32.8 ± 0.2	32.2 ± 0.2	29.2 ± 0.3	20.5 ± 0.2	29.4 ± 0.0	15.9 ± 0.4
HHV (MJ/kg)	17.0 ± 0.3	17.0 ± 0.2	18.6 ± 0.1	17.2 ± 0.6	19.8 ± 0.1	17.7 ± 0.1	19.5 ± 0.0	18.8 ± 0.6	20.8 ± 0.5	20.9 ± 0.1	19.4 ± 0.0	19.5 ± 0.3	21.0 ± 0.1	20.5 ± 0.2	20.0 ± 0.2	15.9 ± 0.4

2   Proximate and ultimate analyses of the harvested biomass during mass cultivation

3   *O1: Open pond raceway #1, O3: Open pond raceway #3

Biodiesel quality analysis of the harvested biomass during mass cultivation

Table 2: Biodiesel quality analysis of the harvested biomass during mass cultivation

1   Table 2:

	APR		MAY		JUN		JUL		AUG		SEP		OCT		NOV		EN 14214	ASTM D6751
	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3	O1	O3		
SV	205.5	202.7	203.5	205.6	205.7	202.5	202.5	201.5	200.9	203.0	202.4	202.3	204.2	199.0	204.1	198.1	-	-
IV (g I_2 100 g^{-1} fat)	63.3	121.2	90.2	58.3	67.4	58.3	68.1	84.17	92.4	106.3	108.7	111.1	94.6	125.8	102.0	112.2	≤ 120	-
CN	60.8	49.2	55.2	49.2	59.6	62.6	60.2	57.5	56.0	52.1	51.8	51.4	54.1	49.2	52.5	52.6	≥ 51	≥ 47
DU (wt %)	52	66	67	52	55	53	55	62	69	69	71	74	69	87	64	82	-	-
LCSF (wt %)	4.1	4.6	3.6	5.1	3.8	4.6	4.2	4.8	4.8	4.1	4.3	4.3	3.9	3.4	3.6	3.6	-	-
CFPP (°C)	−3.7	−2.2	−5.2	−0.5	−4.4	−2.0	−3.4	−1.3	−1.3	−3.7	−2.9	−3.1	−4.3	−5.9	−5.2	−5.3	≤5/≤−20	-
Oxidation stability (h)	24.5	10.0	10.7	18.9	17.4	17.3	12.2	11.9	10.5	9.2	9.1	8.8	9.5	7.8	10.3	9.4	≥ 6	≥ 3
Kinematic viscosity (υ) (mm^2 s^{-1})	4.0	3.8	3.9	3.8	4.0	4.2	4.1	4.1	4.0	3.9	3.9	3.9	3.9	3.9	3.8	4.0	3.5−5.0	1.9−6.0
Density (ρ) (g cm^{-3})	0.87	0.88	0.88	0.88	0.87	0.87	0.87	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.86−0.90	0.82−0.90

2   Biodiesel quality analysis of the harvested biomass during mass cultivation

3   *O1: Open pond raceway #1, O3: Open pond raceway #3