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1 ***In-situ* disinfection and a new downstream processing scheme from algal harvesting to**
2 **lipid extraction using ozone-rich microbubbles for biofuel production**

3

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14

15 **Keywords:**

16 Algal biomass, algal biofuel downstream process, microflotation, microbubble, ozonation,
17 algal lipid extraction.

18

19 **Abstract**

20

21 The scaling up and downstream processing costs of biofuels from microalgae are major
22 concerns. This study focuses on reducing the cost by using energy efficient methods in the
23 production of microalgae biomass and the downstream processes (biomass harvesting and
24 lipid extraction). Ozonation of *Dunaliella salina* (green alga) and *Halomonas* (Gram-negative
25 bacterium) mixed cultures for 10 minutes at 8 mg/l resulted in a reduction in the bacterial

26 contaminant without harming the microalgae. Harvesting of *Dunaliella salina* cells through
27 microflotation resulted in a 93.4% recovery efficiency. Ozonation of the harvested
28 microalgae for 60 minutes produced three main saturated hydrocarbon compounds (2-
29 pentadecanone, 6, 10, 14-trimethyl, hexadecanoic acid, and octadecanoic acid) consisting of
30 16 to 18 carbons. By systematically switching the carrier gas from CO₂ to O₃, the
31 microbubble-driven airlift loop bioreactor (ALB) delivers ~~on-nutrient to the~~ culture and *in-*
32 *situ* disinfection respectively. Further, modulating the bubble size to match particle size
33 ensures recovery of the cells after culture. All three key operations (disinfection, harvesting
34 and lipid extraction) are assembled in a scalable, relatively energy efficient process.

35

36 1. Introduction

37

38 Over the past decade, the majority of the research on sustainable, environmentally
39 friendly energy sources has focused on biofuels. However, pharmaceuticals and
40 nutraceuticals are other crucial co-products in addition to biofuels that are obtainable from
41 microbial biomasses [1]. The production of biofuels and their associated co-products from
42 microalgae basically consists of three main unit operations: culturing (including sterilisation),
43 harvesting (including dewatering) and lipid extraction. All of these operations are largely
44 uneconomical due to the high energy cost of processing [2]. Harvesting and extracting lipids
45 from the microalgal biomass are the most expensive processes. The cost of harvesting itself
46 contributes up to 30% of the cost of the entire process [3]. Brentner et al. [4] has reported that
47 the process of microalgal biomass harvesting through centrifugation, press filtration,
48 supercritical CO₂ and ultrasonication requires 90%, 79%, 66% and 110% of the total energy
49 gained from the biofuel production, respectively.

50 For biofuels to be sustainable, current practices must seek to increase the production

51 efficiency of all key unit processes and increase the profitability of integrated processing
52 plants with co-products. First, algae are known to thrive within a given level of dissolved
53 carbon dioxide and generally grow faster at higher dissolved CO₂ levels [5]. Conversely, the
54 presence of oxygen (a metabolic by-product) can adversely limit growth at high
55 concentrations. Conventional systems typically achieve mixing using motorized impellers or
56 sparge CO₂ into the bioreactors using perforated membranes or pipes; in contrast, little
57 attention has been paid to the bubble size and the resulting hydrodynamic effects on the
58 microbial consortia. However, the resulting hydrodynamic effects can be deleterious to the
59 microbial consortia [6].

60 The successful production of the microalgal biomass is hugely dependent on an
61 axenic (bacteria-free) culture. However, ensuring contaminant-free cultivation can prove
62 challenging because conventional methods can be ineffective. Ozone is one the most widely
63 used disinfectants employed to effectively regulate smell, taste and biological growth and
64 eradicate pigments [7]. Khadre et al. [8] also demonstrated the application of ozone as a
65 powerful antimicrobial agent for food processing and to decontaminate food contact surfaces,
66 equipment and environments. Prior to conversion to useful end-products, the biomass is
67 harvested from cultures and dewatered. Several methods to achieve this goal exist, including
68 filtration, centrifugation and flotation. Recovery by flotation is the industry's most effective
69 technique for colloidal particle recovery. The process entails generating bubbles that attach to
70 the cells and results in the rise of the consortium to the surface of the column, where
71 skimming is performed [9][10].

72 The application of gas bubbles in liquid media is gaining widespread use across many
73 fields, including the above-mentioned operations. Due to their high surface area to volume
74 ratio, microbubbles can be effectively applied in an algal culture to substantially enhance the
75 CO₂ dissolution rates. Introducing microbubbles enriched in CO₂ with negligible oxygen

76 content at the bottom of an algal production tank will alleviate both of these limiting transfer
77 rates [11]. The microbubbles will create a rapid influx of CO₂ and simultaneously extract
78 dissolved oxygen due to the high mass transfer coefficient and oxygen gradient between the
79 phases, so that the bubbles bursting at the top surface completely bypass the boundary layer
80 limitations [11]. This unique property of microbubbles can equally be exploited for culture
81 sterilization and disinfection using ozone. These effects are strongly influenced by the size of
82 the bubbles. Conventional means are relatively inefficient in making small bubbles and often
83 settle for millimeter-sized bubbles. In rare instances when microbubble production is
84 successful, it is not cheap [12]. The typical mechanisms all add external fields with high
85 energy density. In dissolved air flotation for example, pressure levels of 6-8 bars are
86 employed, including the use of saturators to make microbubbles. These combined with the
87 vacuum pumps required to push water into saturators, further increases both the capital and
88 operating costs associated with microbubble production [13]. With the fluidic oscillation
89 approach by contrast, only air, approximately 1000 times less dense than water, is pushed at
90 less friction loss than steady flow through the same piping [11]. So the energy efficiency is a
91 crucial benefit. But capital efficiency is nearly as important as only a low pressure blower is
92 required rather than a compressor. These capital and electricity savings are replicated on just
93 about any scale.

94 This paper reports the development of a novel airlift loop bioreactor where the
95 microbubble dispersal can be switched from a nutrient gaseous input (i.e., CO₂-rich stack gas)
96 to air blown through the plasma reactor to disperse ozone. Furthermore, by only tuning the
97 bubble size, the rig is readily adaptable to harvesting the algae. The expectation is that the
98 algae will grow to a greater density and exhibit higher growth rates with intermittent
99 disinfection but will also be sufficiently axenic to address the high demand for secondary
100 metabolites and lipids for the pharmaceutical and biofuel industries. We believe that our

101 novel process can contribute to *in-situ* disinfection and the development of a cost-efficient
102 disruption method that can be applied on an industrial scale. Therefore, the aims of this paper
103 are to report: (1) the development of axenic conditions at the beginning of the process and
104 intermittent disinfection during the growth phase to eliminate or reduce contamination and
105 (2) the development of an easy and cheap disruption and lipid extraction method using
106 ozonation that is applicable to a large scale.

107

108 **2. Materials and methods**

109

110 ***2.1. Microalgae culture and bioreactor set-up***

111

112 The *Dunaliella salina* strain 19/30 used in the study was obtained from the Culture
113 Centre of Algae and Protozoa, Oban, UK. The culture was grown for 14 days in 250 mL
114 shake flask with 100 mL of working volume during preparation of inoculum. While for the
115 mass production, the strain was grown in 2 L photobioreactor with 1.5 L working volume and
116 both cultivation using artificial seawater as the culture medium [11]. A 10% (v/v) inoculum
117 size (14 days old) was used in all *D. salina* culturing process. A mixture of 5% CO₂ and 95%
118 N₂ was directed into the photobioreactor for 30 minutes every day to serve as a carbon source
119 and agitation. Continuous illumination of the shake flasks and photobioreactors culture were
120 accomplished using a fluorescent lamp at 90 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$; this measurement was
121 obtained using a quantum sensor (Hansatech Instrument Ltd., UK). The experimental set up
122 was based on the previous studies [14]. The *D. salina* culture were maintained at room
123 temperature around 23-25°C.

124 Generally, there were 4 different bioreactors employed in this study (Fig. S1 in
125 supplementary material). Firstly, in the study of contaminant effects on algal growth

126 performance, 0.1 L small bioreactor was used to perform the 10 min ozonation. Secondly, 2 L
127 airlift loop bioreactor (ALB) was used to study the different gas flowrate effects towards *D.*
128 *salina* growth performance. Then, 1 L of microfloatation bioreactor was used during
129 harvesting of the algal cells. Lastly, the cell disruption and lipid extraction was performed in
130 0.15 L ozonation extraction bioreactor. The piping and instrumentation schematic for the
131 novel bioreactor rig consist of the airlift loop bioreactor (ALB) and microfloatation units is
132 shown in Fig. 1. While the processing scheme from cultivated microalgae to algal lipid
133 extraction ~~was~~is illustrated in Fig.2.

134

135 **2.2. Screening of contaminants and disinfection efficiency**

136

137 The screening and isolation of contaminants was accomplished using two methods:
138 the spread plate and streak plate techniques. First, a 100 μ L sample was collected from an old
139 microalgal culture (>3 months) and transferred onto a 1 M NaCl nutrient agar plate. The
140 purpose of using 1 M NaCl in the nutrient plate agar is to simulate the high saline condition
141 of the *D. salina* growth medium. Then, the sample was spread evenly using a glass spreader.
142 The plates were incubated in a 25°C growth room for 3 to 4 days. Visible contaminants were
143 transferred onto new fresh plates via the streak technique to allow the identification of
144 contaminants.

145 The disinfection efficiency of *Halomonas* culture was performed by mixing 50 mL of
146 *Halomonas* culture (5 days old) with 50 mL *D. salina* culture (14 days old) in 0.1 L small
147 bioreactor and ozonated for 10 min. Five mL samples was taken for chlorophyll content
148 analysis while 100 μ L (after serial dilutions) was pipetted onto agar plat and left in the dark
149 area at room temperature for 3-5 days. The disinfection efficiency of *Halomonas* bacteria was
150 determined by counting colony forming units (CFU) on the initial nutrient agar spread plates

151 containing 1 M NaCl. The experiments including the controls were conducted in triplicate.
152 Finally, the disinfection efficiency was calculated by the following equation:

$$S(\%) = \frac{N_0 - N}{N_0} \times 100$$

154

155 Where N_0 and N are the numbers of bacterial colonies (CFU) before and after ozonation,
156 respectively.

157

158 **2.3. Identification of bacterial contaminants**

159

160 Identification of bacterial contaminants was achieved using 16S rRNA gene
161 sequencing. DNA was extracted using the Qiagen DNA purification kit. The PCR was
162 performed using 16S gene universal primers (forward, AGAGTTTGATGCTCAG and
163 reverse, GGTTACCTTGCGACTT). The sequencing was performed by Eurofins Genomics
164 (<http://www.eurofinsgenomics.com>). A BLAST (Basic Local Alignment Search Tool) search
165 (blast.ncbi.nlm.nih.gov) was performed using the obtained partial 16S rRNA sequence as a
166 query against the complete ribosomal database project library.

167

168 **2.4. Chlorophyll content and specific growth rate determination**

169

170 A 15 ml Falcon tube containing a 5 ml microalgal sample was subjected to **full-speed**
171 centrifugation (Hettich Universal 320, UK) at **15000** rpm for ten minutes. After the
172 supernatant was discarded, the pellet was resuspended in 1 ml of distilled water.
173 Subsequently, 4 ml of acetone was added to every tube and adequately mixed by vortexing.
174 The tubes were subjected to full-speed centrifugation for five minutes, and the process was
175 repeated until the pellet became entirely white. The spectrophotometer was zeroed using

176 acetone prior to the measurement of the supernatant's optical density at 645 nm and 663 nm.
177 The experiments including the controls, were conducted in triplicate. The chlorophyll content
178 was calculated using the following equation:

179

$$180 \quad \text{Chlorophyll concentration } (\mu\text{g/mL}) = \frac{OD_{645} \times 202 + OD_{663} \times 80.2}{2 \times 5}$$

181

182 The specific growth rate (μ) was calculated based on method described by Levasseur et al.
183 [15]. The μ was calculated using the following equation:

184

$$185 \quad \text{Specific growth rate } (\mu) = \frac{\ln(c_2/c_1)}{(t_2 - t_1)}$$

186

187 Where c_1 and c_2 are chlorophyll concentrations at time intervals t_1 and t_2 .

188

189 **2.5. The growth performance of contaminated culture with and without ozonation**

190

191 The investigation of the impact of the contaminant on biomass development was
192 conducted using 250 mL shake flask cultures with 100 mL working volume and 10% (v/v)
193 inoculum size. *Halomonas* bacteria 3 days old (approximately 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6
194 and 5×10^6 CFU representing concentrations of 2%, 4%, 6%, 8% and 10% (v/v)) were used.
195 After the introduction of the bacterial contaminant (depending on the concentration) with
196 10% (v/v) 14 days old *D. salina* in 100 mL culture media at the beginning of the experiment,
197 the heterogeneous culture (*D. salina* and *Halomonas*) was left to develop for 14 days at room
198 temperature (23-25 °C). The continuous illumination of the cultures were accomplished using
199 a fluorescent lamp at $90 \mu\text{mol quanta m}^{-2} \text{ s}^{-1}$. On the other hand, for the effect of ozonation

200 towards heterogeneous culture was investigated by bubbling 8 mg/L of ozone for 10 minutes
201 on day one. The ozonation was performed in 0.1 L small bioreactor and conducted in sterile
202 condition. Then, the culture was transferred to 250 mL shake flasks and left to develop (same
203 conditions with heterogeneous culture without ozonation treatment was applied). The
204 experiments including the controls, were conducted in triplicate.

205

206 ***2.6. Microflotation harvesting***

207

208 After accumulation, the *D. salina* biomass was harvested via microflotation [9]. A
209 litre of the *D. salina* culture was obtained (diluted to 1.00 OD₆₈₂) and pretreatment was
210 performed using aluminium sulphate as a coagulant. A flocculator (Stuart, UK) was used to
211 induce rapid mixing at a speed of 250 rpm for 10 min to ensure particle contact with the
212 aluminium sulphate. Thereafter, the mixing speed was reduced to 100 rpm for 5 min to allow
213 the interaction of the particles and the growth of the floc. After this step, the sample was
214 transferred to the one litre microflotation column. According to Hanotu et al. [9]
215 microflotation is a fluidic oscillator-driven system of flotation. The microflotation rig is fitted
216 with a steel mesh diffuser with 50 µm-sized pores. Due to limitation of algal culture, every
217 experiment was run in duplicate for 12 minutes. Samples were collected every 2 minutes to
218 assess the recovery efficiency using the DR 2800 spectrophotometer (HACH Lange, UK) to
219 evaluate the associated absorbance at 682 nm. The algal layer on top of the reactor was
220 scooped out into 50 mL centrifuge tube and stored in 4 °C freezer prior to use in cell
221 disruption and extraction by ozonation.

222

223 ***2.7. Cell disruption and lipid extraction by ozonation***

224

225 Approximately, 5 mL of algal slurries were obtained from every 1 L of *D. salina*
226 culture harvested by microflotation process. The cell disruption process was performed in a
227 0.15 L ozonation bioreactor equipped with a glass diffuser with a pore size of 16-40 µm.
228 Firstly, 10 mL of microalgal slurries were mixed with 20 mL of methanol (1:2 v/v) and the
229 ozonation process was performed at 8 mg/L for 20, 40 and 60 minutes. Due to limitation of
230 algal slurries, the experiments were conducted in duplicate. The air flow rate was 0.1 L/min
231 to ensure that it produced the smallest microbubbles. After the ozonation process, 1 mL of the
232 sample was transferred to 15 mL centrifuge tube. One mL of chloroform was added to the
233 tube and inverted twice (gentle mixing) prior to centrifugation (Hettich Universal 320, UK) at
234 1000 rpm for 10 min to separates the solvent, water and algal cells. The separation method is
235 based on Bligh and Dryer [16] with modification (not to perform solvent-extraction). The
236 bottom layer containing the products in chloroform was transferred to 2 mL centrifuge tube
237 (Eppendorf). The chloroform was evaporated by leaving the tube in the fume hood (air dried)
238 at room temperature (>24 hours). Lastly, 1 mL of methanol was added to dissolve the pellet
239 and transferred to 2 mL glass vial with cap prior to GC-MS analysis. No catalyst (acid) was
240 involved in all steps in order to study the potentials of direct esterification by ozonolysis.

241

242 **2.7.1. Gas Chromatography-Mass Spectrometry (GC-MS)**

243

244 Gas chromatography mass spectroscopy (GC-MS) (AutoSystem XL Gas Chromatograph
245 CHM-100-790, Perkin Elmer) and a TurboMass Mass Spectrometer (13657, Perkin Elmer)
246 fitted with a Zebron ZB-5MS (30 m x 0.25 mm ID x 0.25 µm FT; 7HG-G010-11) GC
247 capillary column were employed to identify the main fatty acids and products present in the
248 ozonated mixture. The GC-MS chromatogram peaks were identified by Perkin Elmer's
249 Turbomass software that linked to a NIST database. Several main compounds detected with

250 high probability (2-pentadecanone, 6, 10, 14-trimethyl, hexadecanoic acid, phytol and
251 octadecanoic acid) were reconfirmed by comparing their retention times to GC-MS standards
252 bought from Sigma Aldrich (UK). The settings highlighted below were used for the analyses:

- 253 • Autosampler method: injection volume: 2 µl; Preinjection solvent washes: 2; Post-
254 injection solvent washes: 6; Split: 20:1; Temperature Program: 60 to 300°C; Ramp 1:
255 2 to 300 °C/min; 20 ml/ min He constant carrier gas flow; MS Scan: EI+; Start mass:
256 50; End mass: 600; Scan time: 0.3 s; Interscan time: 0.1 s; Start time: 0; and End time:
257 100 min.

258

259 ***2.8. Ozone generation and measurement***

260

261 Ozone was generated by a Dryden Aqua ozone generator (corona discharge type)
262 connected by silicone tubing to a glass diffuser type 4 with a pore size of 10-16 µm. To
263 measure the ozone concentration in both the gas and liquid phases, the potassium iodide
264 titration method proposed by Lenore et al. [17] was used. According to Rakness et al. [18],
265 these procedures have previously been used in many water treatment plants and are relevant
266 for all O₃ concentration ranges. Moreover, these iodometric titrations are suitable for both
267 phases, are inexpensive, and the detection limit is dependent on the system [19]. First, ozone
268 gas is maintained at a constant flow rate and passes through a solution containing a certain
269 concentration of potassium iodide. The products react with Na₂S₂O₃ to produce a pale yellow-
270 coloured solution. Then, starch solution is added and a titration is conducted until the blue
271 colour fades. All experiments were conducted in triplicate. Finally, the concentration of
272 ozone is calculated as follows:

273 Ozone concentration (mg/L) =
$$\frac{24 \times V_t \times N_t}{V}$$

274

275 In this case, V is volume of bubble, V_t is volume of sodium thiosulfate used (mL), and N_t is
276 normality of sodium thiosulfate (mg/me).

277

278 ***2.9. Scanning Electron Microscopy (SEM) sample preparation***

279

280 After processing for a period of 3 hours at a temperature of 4 °C in 2-3%
281 | glutaraldehyde in 0.1 M sodium phosphate, the specimens were washed twice at 4 °C in 0.1
282 M phosphate buffer at ten minutes intervals. Then, the specimens were suspended for 1 hour
283 at ambient temperature in 1-2% aqueous osmium tetroxide. Sample dehydration was
284 subsequently undertaken using a consecutive series of ethanol gradients (75%, 95% and
285 100%) for a period of 15 minutes; the samples were left to dry for 15 minutes in 100%
286 ethanol over anhydrous copper sulphate. Afterwards, the specimens were introduced to an
287 equal-part solution of 100% ethanol and 100% hexamethyldisilazane for half an hour and
288 then 100% hexamethyldisilazane for another half hour before being left to dry overnight. The
289 dry samples were affixed onto carbon sticky stubs measuring 12.5 mm in diameter and
290 covered with approximately 25 nm of gold using an Edwards (UK) S150B sputter coater.
291 Finally, the samples were examined in a Philips (UK)/FEI XL-20 scanning electron
292 microscope (SEM) at a 20 KV accelerating voltage.

293

294 **3. Results and discussion**

295

296 ***3.1. Identification of contaminants and its effect to algal growth performance***

297

298 A bacterial contaminant was successfully isolated from an old culture of *D. salina* (>3
299 months) cultivated in a 250 mL shake flask. Sequencing of the 16S rRNA gene showed that
300 the 16S rDNA sequence of the contaminant strain was 100% identical to a group of bacterial
301 strains within the *Halomonas* genus (Table 1). *Halomonas* bacteria are Gram-negative rod-
302 shaped cells that are usually unpigmented or yellow-tinted in colour [20]. These bacterial
303 strains are moderate halophiles (salt loving) and grow well with high levels of NaCl. They are
304 also highly versatile in terms of their ability to successfully grow in a variety of temperature
305 and pH conditions [21]. Previous work has shown that *Dunaliella* cultures are easily
306 contaminated with *Halomonas* bacteria even though the medium contains a high
307 concentration of salt [22].

308 Shake flask cultures (a 100 ml volume of algae in a 250 ml flask) were used to
309 investigate the impact of the contaminant on biomass development. The growth performance
310 of *D. salina* (10% inoculum size) after 14 days of cultivation following contamination with
311 various concentrations of *Halomonas* bacteria is summarized in Table 2 A. After the
312 introduction of the bacterial contaminant at the start of the experiment, the heterogeneous
313 culture was left to develop for 14 days. The results showed that the increase in the
314 contaminant concentration occurred concomitant with a decrease in the algal growth
315 performance. *Halomonas* bacteria (approximately 1×10^6 , 2×10^6 , 3×10^6 , 4×10^6 , and 5×10^6
316 CFU representing concentrations of 2%, 4%, 6%, 8% and 10% (v/v)) resulted in a biomass
317 decrease of 16.3, 29.9, 32.8, 43.9, and 52.9%, respectively. The algal biomass concentration
318 was decreased by over 50% at the 10% (v/v) contaminant concentration, which corroborated
319 the results of earlier research that revealed that bacteria and microalgae were in competition
320 for inorganic nutrients [23]. Zhang et al. [24] reported that microalgae photosynthesis could
321 not occur because the microorganisms and bacterial films covering the internal photo-
322 bioreactor wall reduced the amount of available light. Algae development is hindered by

323 algicidal bacteria directly via cell-to-cell contact or indirectly through extracellular compound
324 secretion [25][26]. This phenomenon was reported when a combination of factors such as
325 nutrient competition, algicidal bacteria, and insufficient light contributed to *C. pyrenoidosa*
326 growth suppression in piggery wastewater exposed to ozonation [27].

327

328 **3.2. Ozonation and characterisation of the heterogeneous culture**

329

330 The effect of ozonation on the heterogeneous culture (*D. salina* and *Halomonas*) was
331 investigated in a 0.1 L bioreactor. Fig. 3 shows the graph of the effect of ozonation of the
332 mixed culture at the 8 mg/L ozone concentration for 10 minutes. Based on the number of
333 microorganisms (CFU), the sterilisation efficiency reached 66% after 5 min and increased to
334 93% after 10 min of ozonation. The chlorophyll concentration can be used to identify the *D.*
335 *salina* cell concentration because ozone can oxidize chlorophyll. The reduction in the
336 chlorophyll concentration suggests that the algal cells are damaged, thus exposing the
337 chlorophyll to ozone attack. The graph shows a reduction in the number of *Halomonas*
338 colonies, whereas the chlorophyll content of *D. salina* remains relatively constant. This result
339 demonstrates that intermittent disinfection can be applied to eliminate or reduce
340 contaminants, with minimal or no damage to the microalgae at the lower ozone concentration
341 over a short period of time. This result is in agreement with the findings of Choi et al. [28],
342 who applied a dielectric barrier discharge (DBD) treatment for more than 1 min to kill
343 *Escherichia coli*, *Bacillus subtilis* and *Pseudomonas* and obtained a sterilisation efficiency of
344 99.99%. Additionally, Gan et al., [27], treated piggery wastewater by bubbling 300 mL/min
345 of ozone gas for 5 min and reported a sterilisation efficiency of 98%.

346 Ozone is a powerful oxidising agent that damages the cell wall, nucleic acids (purines
347 and pyrimidines) and cytoplasmic membrane of the cell, thereby rapidly killing the

348 microorganisms [27][29]. According to Pascual et al., [30], ozone causes inactivation of
349 microbial cells by disrupting their cell membranes or cell lysis by disintegration of the cell
350 walls. Thanomsub et al., [31] proposed that ozone inactivates bacterial cells by destroying
351 cell membranes, leading to cell lysis. However, Cho et al., [32] suggested that inactivation
352 was mainly due to damage to cell surfaces. To achieve sterile conditions such as those
353 obtained with an autoclave, approximately six (6)-log reductions are required [33]. However,
354 this process will kill the entire microbial consortium, including the microalgae. Thus,
355 determining the optimum conditions between the ozone concentration and time is important
356 to reduce the contamination with a minimal or no effect on the microalgal cells.

357 Exposure of 14 days old *D. salina* culture to ozone for a relatively long period of time
358 (>60 minutes) completely destroyed the microalgal cells. Microscopic study revealed that the
359 *D. salina* cells burst and released their intracellular organelles into the culture media. This
360 result was in agreement with Sharma et al. [34], who showed that O₃ treatment on
361 *Microcystis aeruginosa* caused a discharge from within the cells due to harm to the cell wall.
362 Fig. 4 shows morphology of the *D. salina* cells before and after cell disruption executed by
363 ozonation, examined by light microscope. These images clearly showed that the *D. salina*
364 cells were ovoid, green and healthy prior to ozonation (Fig. 4A). After 30 minutes, the sizes
365 of the cells were distorted and shrunk, and some were completely damaged (Fig. 4B).
366 Moreover, ozonation for 60 minutes resulted in dramatic shrinkage of the anterior
367 cytoplasmic compartment of the cells, whereas the posterior chloroplast still looked largely
368 intact (Fig. 4C). However, the *D. salina* cells were totally disrupted and colourless after 90
369 minutes of ozonation, which indicated that the cell contents were released into the culture
370 media and probably oxidized (Fig. 4D). The SEM images in Fig. 5 show normal cell
371 structures versus damaged cells due to the ozone treatment. The cultivation of algae and the
372 extraction of its metabolites are significantly affected by the concentration of ozone and the

373 competitive reactions among the organic substances and toxins. Hammes et al., [35] reported
374 that treatment of the algal mass with ozone caused the release of extracellular organic
375 substances.

376 In order to study the improvement of ozonation treatment towards contaminated
377 culture, the heterogeneous cultures were ozonated with 8 mg/L of ozone concentration for 10
378 minutes on day one and the results is summarized in Table 2 B. The production of the
379 microalgal biomass at increased microbial contamination concentrations of 2%, 4%, 6%, 8%
380 and 10% (v/v) of *Halomonas* resulted in a biomass reduction of 4.8%, 7.9%, 10.1%, 21.4%
381 and 28.6%, respectively. The results shows that the ozonation at the beginning of experiment
382 can control the contamination as compared to without ozonation thus slightly increased the
383 biomass production. It has been reported that ozonation at 2.59 to 3.11 mg/L was associated
384 with excellent disinfection effects that were able to suppress the growth of bacterial cells and
385 their spores [36]. Although a 93% efficiency of disinfection was attained within 10 minutes
386 of ozonation, the growth of residual bacterial persisted along with the growth of the
387 microalgae. These results agree with those obtained by Gan et al. [27], whose study focused
388 on the use of ozone for the treatment of piggery wastewater and recorded a 98% efficiency of
389 disinfection attained after a five-minute ozonation process at 8 mg/L. The study also recorded
390 the growth of residual bacteria along with the microalgae in the piggery wastewater.

391

392 3.3. CO₂ flowrate effect on *Dunaliella salina* culture

393

394 The second of the cultivation experiments was completed over a longer period of time
395 and with three cultivations for each parameter. Here the effect of 5% CO₂ and 95% N₂ gas
396 flow rate on algal growth assessed is illustrated in Fig 6. The selected flow rates include 0.1,
397 0.3, 0.5, 0.7 and 0.9 L/min. Two liter airlift bioreactors (ALB) with 1.5 L working volume
398 were employed in the experiment. The results of the experiment demonstrated that the ALB

399 attained a higher accumulation of biomass compared to the bubble column bioreactor within
400 25 days of culture. The bubble column at the optimum algal concentration attained a
401 chlorophyll content of 33.4 mg/L, whereas the airlift loop at the same flow rate (0.5 L/min)
402 highly exceeded this concentration by attaining an optimum concentration of 42.9 mg/L,
403 which represented a 28.4% increment. The maximum concentration of chlorophyll (54.78
404 mg/L) was obtained at a flow rate of 0.9 L/min, representing a 55.4% increase compared to
405 the lowest concentration attained at the 0.1 L/min flow rate (35.25 mg/L).

406 **In general, the specific growth rate (μ) of *D. salina* culture grown in ALB were**
407 increased with the increment of gas flow rate starting from 0.1 L/min (0.23 day⁻¹), 0.3 L/min
408 (0.24 day⁻¹), 0.5 L/min (0.29 day⁻¹), 0.7 L/min (0.31 day⁻¹) and 0.9 L/min (0.31 day⁻¹),
409 respectively. Both 0.7 and 0.9 L/min showed 25% improvement as compared to the lowest
410 (0.1 L/min). However the culture grown in bubble column with 0.5 L/min showed higher
411 specific growth rate (0.28 day⁻¹) as compared to culture grown in ALB with 0.1 and 0.3
412 L/min gas flowrate. The airlift loop bioreactor fitted with microbubble dosing allowed a high
413 mass transfer of carbon dioxide dissolution and oxygen elimination [37]. A study by
414 Zimmerman et al., [11] showed a pilot scale microalgal culture (2200 L) similarly designed to
415 the type used in this study, revealed that the ALB culture was neither carbon dioxide-limited
416 nor oxygen-inhibited, resulting in a high growth rate of the algal cells. According to a study
417 conducted by Ying et al., [37] an optimum rate of growth (μ) of *D. salina* was achieved in
418 their slightly larger 3 L airlift loop bioreactor fitted with a fluidic oscillator at a 0.9 L/min
419 flow rate. Nevertheless, the rate of growth was abruptly reduced by increasing the flow rate
420 up to 1.1 L/min. This result was attributed to the production of a high degree of turbulence
421 that caused damage to the algal cells. Surprisingly, the maximum chlorophyll content gained
422 (32.65 mg/L) at a 0.9 L/min flow rate in their study was far lower than recent finding (54.78
423 mg/L). This can be due to different algal cells concentration introduced during the inoculation

424 process. Apart from that, the contamination during inoculum preparation or during cultivation
425 itself might possibly be the reason as the cultivation was performed at open space. Thus, the
426 axenic conditions during cultivation should be prioritised to prevent great loss of algal
427 biomass.

428

429 **3.4. Microalgae harvest by Microflotation**

430

431 In addition to the advantageous mass transfer properties discussed above,
432 microbubbles have important and useful momentum transfer and coordination properties.
433 Microbubbles can attach to algal cells, giving the whole complex greater buoyancy. Such
434 flotation brings the flocculated algal cells to the surface where they are more readily
435 harvested by skimming. Fig. 7 shows the effect of different coagulant concentrations over
436 time. The lowest recovery efficiency obtained was 44.6% at 300 mg/L, followed by 71.3% at
437 400 mg/L, 84.1% at 500 mg/L, 88.9% at 600 mg/l and 93.4% at 700 mg/L. The result shows
438 an increase in the recovery of microalgal cells as the concentration of the coagulant increases.
439 This increase is due to compression of the double layer effect, which is essential for the
440 agglomeration of particles within the isoelectric point; thus, increasing the dosage of the
441 coagulant provides more of the trivalent ions necessary for double layer compression [38].
442 The agglomerated cells readily attach to the rising microbubbles and are transported to the
443 top of the floatation reactor-separator for collection. This result is in agreement with Hanotu
444 et al. [9], who obtained higher recovery efficiency, using a similar set-up.

445

446 **3.5. Microalgal lipid extraction and yields**

447

448 The harvested microalgal biomass (known as algal slurries) was then ozonated in a
449 0.15 L ozonation bioreactor to extract the lipids from the cells. Fig. 9 shows GC-MS
450 chromatograms of compounds detected after the ozonation process. While the chemical
451 compounds with highest probability based on NIST Database is summarized in Table 3.
452 Ozonation of the mixture for 20 minutes produced several compounds (2-pentadecanone, 6,
453 10, 14-trimethyl, n-hexadecanoic acid (also known as palmitic acid), phytol and octadecanoic
454 acid (also known as stearic acid)). Ozonation for 40 and 60 minutes clearly produced 3 main
455 compounds (2-pentadecanone, 6, 10, 14-trimethyl, palmitic acid and stearic acid). Due to the
456 sensitiveness of polyunsaturated fatty acid to oxidation [39], we can observe the
457 accumulation of saturated fatty acid (hexadecanoic acid, octadecanoic acid) incoherent with
458 the time of ozonation. Lin and Hong [42] reported that ozonation of *Chlorococcum*
459 *aquaticum* with methanol in a sand filtration reactor generated several products in the forms
460 of long-chain largely saturated hydrocarbons with 16 to 20 carbons. They also suggested that
461 with ozonation, the composition of biodiesel can be controlled and would be beneficial for
462 utilization in cold regions (unsaturated hydrocarbon) and more oxidation resistant (saturated
463 hydrocarbon).

464

465 The control for the present study produced low concentration of hexadecanoic acid
466 and phytol which are due to minimal breakage of the cells during separation process (solvent
467 and centrifugation). Phytol is an acrylic diterpene alcohol which is originated from
468 chlorophyll metabolism and use in industries as fragrance agent (flowery odor) [40]. The
469 increment of 2-pentadecanone, 6, 10, 14-trimethyl, which has been previously reported in
470 *Scenedesmus* and *Chlorella vulgaris* extracted by steam distillation [41], could be due to the
471 degradation of higher hydrocarbon compounds which is in this case, phytol. Fig. 10 shows

472 the possible degradation mechanism of phytol to 2-pentadecanone, 6, 10, 14-trimethyl by
473 oxidation process.

474

475 ~~***The modified Bligh and Dryer method employed in present study was is to limit~~
476 ~~the ability of chloroform to extract interior lipids. Thus, the short contact time is crucial as the~~
477 ~~main purpose was to separate the solvents and the cells, not to extracts more lipids from the~~
478 ~~cells. However, the increase in cell disruption (ozonation), increasing-increases the~~
479 ~~performance-efficacy of the solvent (chloroform). Thus, the claim thated for ozonation~~
480 ~~extraction is solely responsible for the products gained in present study cannot be~~
481 ~~appliedfully supported.~~ Do we need to mention about this? Because the method is mentioned
482 in line 232. “The separation method is based on Bligh and Dryer [16] with modification (not
483 to perform solvent-extraction)”.

484

485 ~~Surprisingly, some trace of methyl ester compound was detected proving that~~
486 ~~ozonolysis process also managed to esterify the fatty acid. (Still looking for explanation and~~
487 ~~references).~~

488

489 Based on the findings, the lipid extraction by ozonation can possibly omit the needs of
490 energy intensive pretreatment methods such as microwave, bead mills, osmotic pressure,
491 autoclave, electroporation, and ultrasonication which previously have been reported to
492 improve the efficiency of the solvent-extraction process [43]. Moreover, ozonation-extraction
493 process might possibly the solution to the solvent diffusion limitation and lipids polarity
494 problems faced during high water content in solvent extraction process [44]. However, more
495 conclusive studies should be conducted as present study was performed to *Dunaliella salina*

496 known to have less rigid cell wall [45]. The results of the lipid content optimization and cost
497 analysis will be reported in a forthcoming manuscript.

498

499 **4. Conclusion**

500

501 A novel microbubble driven photobioreactor system integrating ozonation during the
502 production stage, microflotation during harvesting and lipid extraction by direct ozonation
503 has been developed and tested. The study on the effect of ozone on the algal mixture shows
504 that intermittent disinfection can be applied to eliminate or reduce contaminants. Harvesting
505 using microflotation results in a high recovery efficiency. Ozonation of harvested microalgae
506 in a methanol ruptures the microalgae and extracts the algal lipids, accumulates saturated
507 fatty acid. Overall, these results are readily scalable by essentially matching local bubble flux
508 rates on a large scale.

509

510 **References**

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646

647

648 **List of Figures**

649

650 **Fig. 2:** Processing scheme from cultivated microalgae to algal lipid extraction. A; 2 L airlift
651 loop bioreactor (ALB), B; 1 L microfloatation bioreactor and C; 0.15 L ozone extraction
652 bioreactor

653

654 **Fig. 3:** *Halomonas* colony forming unit (initial number: $2.15 \pm 0.11 \times 10^6$ CFU) and
655 chlorophyll content (*Dunaliella salina*) of the mix culture (1:1 (v/v)) after 10 minutes of
656 ozonation at 8 mg/L. The experiments were conducted thrice for each parameter (triplicate)
657 which represent by the error bar

658

659 **Fig. 4:** Morphology of *Dunaliella salina* after ozonation at 8 mg/L under light microscope
660 (1000x magnification). A; control, B; 30 minutes of ozonation, C; 60 minutes of ozonation
661 and D; 90 minutes of ozonation

662

663 **Fig. 5:** SEM images of *Dunaliella salina* before and after ozonation at 8 mg/L. A; Control,
664 under light microscope (1000x magnification), B; Control, SEM image, C; after 60 min of
665 ozonation, D; after 120 min of ozonation

666

667 **Fig. 6:** Growth performance of *D. salina* culture in a 2 L photobioreactor with different flow
668 rate. A 30 minutes gas (5 % CO₂: 95% N₂) bubbling (depending on flow rate) was
669 performed every day. The experiments were conducted thrice for each parameter (triplicate)
670 which represent by the error bar

671

672 **Fig. 7:** Microflotation harvesting with different aluminium sulphate concentrations. It would
673 have been relevant to test further coagulant concentration to ascertain optimality but given
674 that the priority is demonstrating microflotation performance as a modular unit, the results
675 provide sufficient information. The experiment was conducted twice for each parameter
676 (duplicate) and each point representing the mean of the value

677

678 **Fig. 8:** GC-MS chromatograms of the identified compounds in microalgae extracts collected
679 after the ozonation process

680

681 **Fig. 9:** Degradation mechanism of phytol to 2-pentadecanone, 6, 10, 14-trimethyl by ozone
682 oxidation

683

684 **List of Tables**

685

686 **Table 1:** Top 10 similarity between reverse 16SrRNA gene sequences of contaminant strain
687 and other related strains from the NCBI database

688

689 **Table 2:** The growth performance of heterogeneous culture with and without ozonation. A;
690 The growth performance of *D. salina* with *Halomonas* contamination. B; The growth
691 performance *D. salina* with *Halomonas* contamination treated with ozone

692

693 **Table 3:** The chemical compounds detected with highest probability (NIST Database) and its
694 concentration. Control represent sample without ozonation, while final is sample after 60
695 minutes of ozonation. Compounds 1, 2, and 3 are the highest produced after 60 min of

696 ozonation. The main products were reconfirmed with GCMS standards chemicals (Sigma
697 Aldrich, UK).

698

699 **Supplementary Materials**

700

701 **Fig. S1:** Bioreactor set-up: A; 0.1 L small bioreactor (disinfection), B; 2L airlift loop
702 bioreactor (biomass production), C; 1L microfloatation bioreactor (harvesting), D; 0.15 L
703 ozone extraction bioreactor

704

705 **Fig. S2:** Ozone generator volume control (minimum, medium and maximum)

706

707 **Table S1:** Ozone generator calibration at minimum, medium and maximum volume setting.

708 The medium setting of ozone generator producing $7.68 \pm 0.48 \approx 8$ mg/L was chosen as it
709 produced consistent ozone concentration within 60 minutes

710