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

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Abstract

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

contaminant without harming the microalgae. Harvesting of Dunaliella salina cells through microflotation resulted in a 93.4% recovery efficiency. Ozonation of the harvested microalgae for 60 minutes produced three main saturated hydrocarbon compounds (2-pentadecanone, 6, 10, 14-trimethyl, hexadecanoic acid, and octadecanoic acid) consisting of 16 to 18 carbons. By systematically switching the carrier gas from CO_2 to O_3, the microbubble-driven airlift loop bioreactor (ALB) delivers ~~on~~ nutrient to the culture and in-situ disinfection respectively. Further, modulating the bubble size to match particle size ensures recovery of the cells after culture. All three key operations (disinfection, harvesting and lipid extraction) are assembled in a scalable, relatively energy efficient process.

1. Introduction

Over the past decade, the majority of the research on sustainable, environmentally friendly energy sources has focused on biofuels. However, pharmaceuticals and nutraceuticals are other crucial co-products in addition to biofuels that are obtainable from microbial biomasses [1]. The production of biofuels and their associated co-products from microalgae basically consists of three main unit operations: culturing (including sterilisation), harvesting (including dewatering) and lipid extraction. All of these operations are largely uneconomical due to the high energy cost of processing [2]. Harvesting and extracting lipids from the microalgal biomass are the most expensive processes. The cost of harvesting itself contributes up to 30% of the cost of the entire process [3]. Brentner et al. [4] has reported that the process of microalgal biomass harvesting through centrifugation, press filtration, supercritical CO_2 and ultrasonication requires 90%, 79%, 66% and 110% of the total energy gained from the biofuel production, respectively.

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

efficiency of all key unit processes and increase the profitability of integrated processing plants with co-products. First, algae are known to thrive within a given level of dissolved carbon dioxide and generally grow faster at higher dissolved CO_2 levels [5]. Conversely, the presence of oxygen (a metabolic by-product) can adversely limit growth at high concentrations. Conventional systems typically achieve mixing using motorized impellers or sparge CO_2 into the bioreactors using perforated membranes or pipes; in contrast, little attention has been paid to the bubble size and the resulting hydrodynamic effects on the microbial consortia. However, the resulting hydrodynamic effects can be deleterious to the microbial consortia [6].

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

The application of gas bubbles in liquid media is gaining widespread use across many fields, including the above-mentioned operations. Due to their high surface area to volume ratio, microbubbles can be effectively applied in an algal culture to substantially enhance the CO_2 dissolution rates. Introducing microbubbles enriched in CO_2 with negligible oxygen

content at the bottom of an algal production tank will alleviate both of these limiting transfer rates [11]. The microbubbles will create a rapid influx of CO_2 and simultaneously extract dissolved oxygen due to the high mass transfer coefficient and oxygen gradient between the phases, so that the bubbles bursting at the top surface completely bypass the boundary layer limitations [11]. This unique property of microbubbles can equally be exploited for culture sterilization and disinfection using ozone. These effects are strongly influenced by the size of the bubbles. Conventional means are relatively inefficient in making small bubbles and often settle for millimeter-sized bubbles. In rare instances when microbubble production is successful, it is not cheap [12]. The typical mechanisms all add external fields with high energy density. In dissolved air flotation for example, pressure levels of 6-8 bars are employed, including the use of saturators to make microbubbles. These combined with the vacuum pumps required to push water into saturators, further increases both the capital and operating costs associated with microbubble production [13]. With the fluidic oscillation approach by contrast, only air, approximately 1000 times less dense than water, is pushed at less friction loss than steady flow through the same piping [11]. So the energy efficiency is a crucial benefit. But capital efficiency is nearly as important as only a low pressure blower is required rather than a compressor. These capital and electricity savings are replicated on just about any scale.

This paper reports the development of a novel airlift loop bioreactor where the microbubble dispersal can be switched from a nutrient gaseous input (i.e., CO_2-rich stack gas) to air blown through the plasma reactor to disperse ozone. Furthermore, by only tuning the bubble size, the rig is readily adaptable to harvesting the algae. The expectation is that the algae will grow to a greater density and exhibit higher growth rates with intermittent disinfection but will also be sufficiently axenic to address the high demand for secondary metabolites and lipids for the pharmaceutical and biofuel industries. We believe that our

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

2. Materials and methods

2.1. Microalgae culture and bioreactor set-up

The Dunaliella salina strain 19/30 used in the study was obtained from the Culture Centre of Algae and Protozoa, Oban, UK. The culture was grown for 14 days in 250 mL shake flask with 100 mL of working volume during preparation of inoculum. While for the mass production, the strain was grown in 2 L photobioreactor with 1.5 L working volume and both cultivation using artificial seawater as the culture medium [11]. A 10% (v/v) inoculum size (14 days old) was used in all D. salina culturing process. A mixture of 5% CO_2 and 95% N_2 was directed into the photobioreactor for 30 minutes every day to serve as a carbon source and agitation. Continuous illumination of the shake flasks and photobioreactors culture were accomplished using a fluorescent lamp at 90 µmol quanta m^{-2} s^{-1}; this measurement was obtained using a quantum sensor (Hansatech Instrument Ltd., UK). The experimental set up was based on the previous studies [14]. The D. salina culture were maintained at room temperature around 23-25°C.

Generally, there were 4 different bioreactors employed in this study (Fig. S1 in 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 microflo~~a~~tation 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 microflo~~a~~tation 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 µL 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 µL (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

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

$$S(\%) = \frac{No - N}{No} \times 100 \quad (3)$$

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

2.3. Identification of bacterial contaminants

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

2.4. Chlorophyll content and specific growth rate determination

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

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

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

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

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

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

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

The investigation of the impact of the contaminant on biomass development was conducted using 250 mL shake flask cultures with 100 mL working volume and 10% (v/v) inoculums size. Halomonas bacteria 3 days old (approximately $1x10^6$, $2x10^6$, $3x10^6$, $4x10^6$ and $5x10^6$ CFU representing concentrations of 2%, 4%, 6%, 8% and 10% (v/v)) were used. After the introduction of the bacterial contaminant (depending on the concentration) with 10% (v/v) 14 days old D. salina in 100 mL culture media at the beginning of the experiment, the heterogeneous culture (D. salina and Halomonas) was left to develop for 14 days at room temperature (23-25 ˚C). The continuous illumination of the cultures were accomplished using a fluorescent lamp at 90 μmol quanta m^{-2} s^{-1}. On the other hand, for the effect of ozonation

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

2.6. Microflotation harvesting

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

2.7. Cell disruption and lipid extraction by ozonation

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

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

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

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

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

2.8. Ozone generation and measurement

Ozone was generated by a Dryden Aqua ozone generator (corona discharge type) connected by silicone tubing to a glass diffuser type 4 with a pore size of 10-16 µm**.** To measure the ozone concentration in both the gas and liquid phases, the potassium iodide titration method proposed by Lenore et al. [17] was used. According to Rakness et al. [18], these procedures have previously been used in many water treatment plants and are relevant for all O_3 concentration ranges. Moreover, these iodometric titrations are suitable for both phases, are inexpensive, and the detection limit is dependent on the system [19]. First, ozone gas is maintained at a constant flow rate and passes through a solution containing a certain concentration of potassium iodide. The products react with $Na_2S_2O_3$ to produce a pale yellow-coloured solution. Then, starch solution is added and a titration is conducted until the blue colour fades. All experiments were conducted in triplicate. Finally, the concentration of ozone is calculated as follows:

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

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

2.9. Scanning Electron Microscopy (SEM) sample preparation

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

3. Results and discussion

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

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

Shake flask cultures (a 100 ml volume of algae in a 250 ml flask) were used to investigate the impact of the contaminant on biomass development. The growth performance of D. salina (10% inoculum size) after 14 days of cultivation following contamination with various concentrations of Halomonas bacteria is summarized in Table 2 A. After the introduction of the bacterial contaminant at the start of the experiment, the heterogeneous culture was left to develop for 14 days. The results showed that the increase in the contaminant concentration occurred concomitant with a decrease in the algal growth performance. Halomonas bacteria (approximately $1x10^6$, $2x10^6$, $3x10^6$, $4x10^6$, and $5x10^6$ CFU representing concentrations of 2%, 4%, 6%, 8% and 10% (v/v)) resulted in a biomass decrease of 16.3, 29.9, 32.8, 43.9, and 52.9%, respectively. The algal biomass concentration was decreased by over 50% at the 10% (v/v) contaminant concentration, which corroborated the results of earlier research that revealed that bacteria and microalgae were in competition for inorganic nutrients [23]. Zhang et al. [24] reported that microalgae photosynthesis could not occur because the microorganisms and bacterial films covering the internal photo-bioreactor wall reduced the amount of available light. Algae development is hindered by

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

3.2. Ozonation and characterisation of the heterogeneous culture

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

Ozone is a powerful oxidising agent that damages the cell wall, nucleic acids (purines 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 O_3 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

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

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

3.3. CO_2 flowrate effect on Dunaliella salina culture

The second of the cultivation experiments was completed over a longer period of time and with three cultivations for each parameter. Here the effect of 5% CO_2 and 95% N_2 gas flow rate on algal growth assessed is illustrated in Fig 6. The selected flow rates include 0.1, 0.3, 0.5, 0.7 and 0.9 L/min. Two liter airlift bioreactors (ALB) with 1.5 L working volume were employed in the experiment. The results of the experiment demonstrated that the ALB

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

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

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

3.4. Microalgae harvest by Microflotation

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

3.5. Microalgal lipid extraction and yields

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

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

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

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

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

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

4. Conclusion

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

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650 **Fig. 2:** Processing scheme from cultivated microalgae to algal lipid extraction. A; 2 L airlift
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652 bioreactor

653

654 **Fig. 3:** Halomonas colony forming unit (initial number: 2.15 ± 0.11 $x10^6$ 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

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

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

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

Supplementary Materials

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

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

Table S1: Ozone generator calibration at minimum, medium and maximum volume setting. The medium setting of ozone generator producing $7.68 \pm 0.48 \approx 8$ mg/L was chosen as it produced consistent ozone concentration within 60 minutes