Biocatalysis and Agricultural Biotechnology 23 (2020) 101462

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: http://www.elsevier.com/locate/bab

Optimization and cost estimation of microalgal lipid extraction using ozone-rich microbubbles for biodiesel production

M. Farizal Kamaroddin [a,*], Ameera Rahaman [a], D. James Gilmour [b], William B. Zimmerman [c]

[a] Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310, UTM, Johor Bahru, Malaysia
[b] Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, United Kingdom
[c] Department of Chemical and Biological Engineering, University of Sheffield, Mappin Street, Sheffield, S1 3JD, United Kingdom

ARTICLE INFO

Keywords:
Algal biodiesel downstream process
Ozone-rich microbubbles
Algal lipid extraction

ABSTRACT

Harvesting and extracting lipids from the microalgal biomass are the most expensive processes in biodiesel production. This study focuses on reducing the lipid extraction cost using ozone-rich microbubbles technique. The lipid extraction of *Dunaliella salina* slurry with methanol (1:2 v/v) was performed in a 0.2 L bioreactor at room temperature with direct ozonation (8 mg L^{-1}). When the temperature was increased (60 °C) and smaller bubbles were introduced during extraction, the concentration of products increased significantly to around 156%, 88.9% and 150% for 6,10,14-trimethylpentadecan-2-one, palmitic acid and stearic acid, respectively. The energy usage for extracting *D. salina* lipid with ozone has been estimated to be around 2.16 MJ kg^{-1} dry algae (36% energy) which is a small fraction of the energy that is used in the production of biodiesel, unlike centrifugation and solvent extraction methods, which consume more than 90% of the energy.

1. Introduction

Over the past decade, the majority of the research on sustainable, environmentally friendly energy sources has focused on biofuels (Salam et al., 2016). This due to an expanding demand of fossil fuels and people are searching for alternative to petroleum products by developing microalgae-based biodiesel (Sivaramakrishnan et al., 2019; Mathimani and Pugazhendhi, 2019). Apart from that, pharmaceuticals and nutraceuticals are other crucial co-products in addition to biodiesels that are obtainable from microbial biomass (Harun et al., 2010). The production of biodiesel 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 (Brennan and Owende, 2010). For biodiesels 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.

Harvesting and extracting lipids from the microalgal biomass are the most expensive processes (Mubarak et al., 2015). The cost of harvesting itself contributes up to 30% of the cost of the entire process (Kim et al., 2013). Brentner et al. (2011) has reported that the process of microalgal biomass harvesting through centrifugation and press filtration, requires 90% and 79% of the total energy gained from the biodiesel production, while lipid extraction through supercritical CO_2 and ultrasonication requires 66% and 110%. It is economically acceptable if the extracted compounds are high value and low volume products (pharmaceutical industry) (Salam et al., 2016). However, it is not sustainable if the compounds (lipids) are solely extracted for biodiesel production (Surendhiran and Vijay, 2014). Therefore, a more energy efficient algal lipid extraction method should be utilised in order to make the biodiesel economically sustainable.

According to Chisti (2007), the major importance of algal cultivation is in the transformation of algal lipids into biodiesel, which is the lipid extraction step following on from the algal biomass dehydration stage. Efforts for the development of alternatives or improvement of this infrastructure are currently underway. Traditionally, the extraction of lipids has been based on mechanical principles such as disruption alongside the solvent fractionation, but these procedures waste a lot of biomass and are slow (Halim et al., 2012). Great potential has been illustrated by the latest approaches such as electroporation, which consume energy efficiently in addition to producing higher outputs (Halim et al., 2012). Lam and Lee (2012), point out that a mechanical press of the type that is effective in extracting oil for soil-grown crops is

* Corresponding author.
E-mail address: farizal@utm.my (M.F. Kamaroddin).

https://doi.org/10.1016/j.bcab.2019.101462
Received 5 August 2019; Received in revised form 29 October 2019; Accepted 4 December 2019
Available online 9 December 2019
1878-8181/

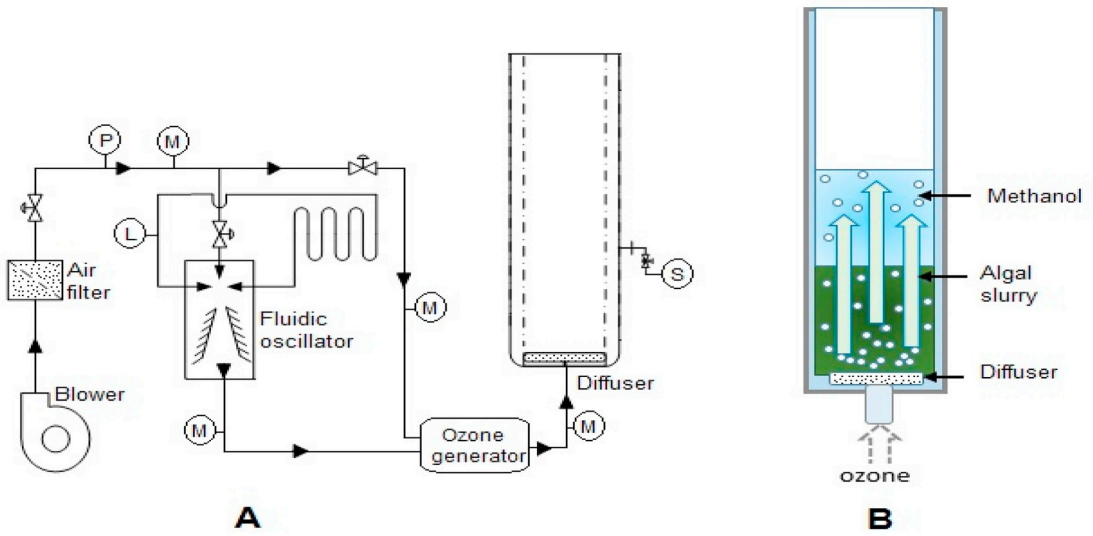

Fig. 1. Processing scheme from cultivated microalgae to algal lipid extraction. 0.2 L ozone extraction bioreactor.

not likely to be effective for lipid extraction from algal biomass because the presence of the thick cell wall impedes the release of intracellular lipids.

Halim et al. (2012), argue that an optimal methodology for algal lipid extraction must be strongly specific and selective with respect to algal lipids such as acylglycerol without resulting in the concurrent extraction of other compounds (proteins, carbohydrates, ketones, carotenes) that are not useful for conversion to biodiesel. The fact that chemical solvent displays strong specificity toward algal lipids, along with the solubility of algal lipids in such solvents, makes this common laboratory-scale method the most useful methodology for algal lipid extraction. According to Halim et al. (2012) and Ranjan et al. (2010), this methodology even facilitates diffusion of intracellular lipids across the algal cell walls, allowing these to be successfully extracted. Halim et al. (2012) and Lam and Lee (2012), list solvents including n-hexane, methanol, ethanol, and mixed polar/nonpolar chemical solvents such as methanol/chloroform and hexane/isopropanol as being applicable to algal lipid extraction, although the effectiveness of the extraction is still strongly conditional on the algal strain.

A number of problems must be resolved before chemical solvent extraction can be commercially employed. These include the necessary use of significant amounts of chemical solvent for practical lipid extraction, the health and safety considerations relating to the toxicity of these solvents, the further energy consumption required for solvent recovery, and further costs arising from the need for wastewater treatment. Lee et al. (2010), have described autoclaving as a possible alternative technology for enhanced algal lipid extraction; Halim et al. (2012) suggest the application of supercritical CO_2, while Adam et al. (2012), Lee et al. (2010) and Prabakaran and Ravindran (2011), suggest ultrasonication. All of these techniques remain in the research stage (lab scale) and there is a pressing need for them to be fully optimized before they can be commercially applied.

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 mass production of culture and the harvesting technique used to obtain the algal slurries was reported in the previous study (Kamaroddin et al., 2016). The cell disruption and lipid extraction equipped with 16 μm sintered glass diffuser was performed in 0.2 L ozonation extraction bioreactor. The piping and instrumentation schematic for the novel bioreactor rig and the processing scheme from cultivated microalgae to algal lipid extraction is illustrated in Fig. 1.

2.2. Chlorophyll content estimation

A 15 mL Falcon tube containing a 5 mL microalgal sample was subjected to centrifugation (Hettich Universal 320, UK) at 3000 rpm for 10 min to separate the cells. The pellet was resuspended in 1 mL of distilled water after the supernatant had been discarded. Subsequently, 4 mL of acetone was added to every tube and adequately mixed by vortexing. The tubes were subjected to full-speed centrifugation at 15,000 rpm for 5 min, 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 (OD_{645} and OD_{663}). The experiments including the controls, were conducted in triplicate. The chlorophyll content was calculated using the following equation:

$$\text{Chlorophyll concentration}\left(\mu g \Big/ mL\right) = \frac{OD645 \times 202 + OD663 \times 80.2}{2 \times 5}$$

2.3. Cell disruption and lipid extraction by ozonation

The cell disruption process was performed in a 0.2 L ozonation bioreactor equipped with a glass diffuser with a pore size of 16 μm which can produce microbubbles at an average size of 46 μm in diameter at 0.1 L min^{-1} of air (Kokoo, 2015). In aqueous solution, the solubility of ozone is relatively unstable. Its decomposition to oxygen in aqueous solution is continuous but slow, based on a pseudo first-order reaction (Tomiyasu et al., 1985). Thus, the harvested algal slurries were mixed with methanol during the extraction process. 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^{-1} for 20, 40 and 60 min. Due to limited availability of algal slurries, the experiments were conducted in duplicates. The air flow rate was 0.1 L min^{-1} to ensure that it produced the smallest microbubbles. After the ozonation process, 1 mL of the sample was transferred to a 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 separate the solvent, water and algal cells. The separation method is based on Bligh and Dryer (1959), with modifications to limit the extraction to easily accessible lipids. The bottom layer containing the products in

Table 1
The standard chemicals used for product confirmation.

Compound detected (NIST database)	Molecular formula	Group	Standard chemicals (confirmation)
1-Hexadecene $C_{16}H_{32}$	$C_{16}H_{32}$	Alkene	Sigma Aldrich, UK
8-Heptadecene + isomers	$C_{17}H_{34}$	Alkene	Sigma Aldrich, UK
Hexadecanoic acid $C_{16}H_{32}$	$C_{16}H_{32}O_2$	Fatty acid	Sigma Aldrich, UK
2-Pentadecanone-6,10,14-trimethyl	$C_{18}H_{36}O$	Hexahydrofarnesyl acetone	Sigma Aldrich, UK
Phytol + isomers	$C_{20}H_{40}O$	Acrylic diterpene alcohol	Sigma Aldrich, UK
Octadecanoic acid	$C_{18}H_{36}O_2$	Fatty acid	Sigma Aldrich, UK

chloroform was transferred to a 2 mL centrifuge tube (Eppendorf). The chloroform was evaporated by leaving the tube in the fume hood (air dried) at room temperature (>24 h). However, drying under nitrogen is recommended and will be applied in future studies as air drying may lead to some oxidation problems. Lastly, 1 mL of methanol was added to dissolve the pellet and transferred to a 2 mL glass vial with cap prior to GC-MS analysis. No catalyst (acid) was involved in any step, in order to study the potential of direct esterification by ozonolysis.

2.4. Algal lipid transesterification

Algal direct transesterification was performed based on the methods suggested by Griffiths et al. (2010) with slight modification. The ozonated sample from Method 2.3 was added with 190 μl chloroform/methanol (2:1, v/v) to solubilise the lipid. Internal standard for reaction efficiency purposes containing 10 μl (0.1 mg) tridecanoic acid, C13 lipid were added. Followed by the addition of 0.3 mL HCl/MeOH (5%, v/v) catalyst, crimp-sealed quickly and mixed gently by hand. The vials were placed on 85 °C hot plate for 1 h to transesterifying the lipids. After cooled it to room temperature for 5 min, 975 μl of Hexane were added, mixed well and left for 1 h (room temperature) for FAME extraction. 487.5 μl of the top hexane phase were transferred into a fresh vial with the addition of 12.5 μl (0.125 mg) of known compound as control (tetradecene) prior to vial sealed. Then, the vial containing FAMEs were quantified and identified by GC-MC analysis.

2.5. Gas chromatography-mass spectrometry (GC-MS)

The identification of the main fatty acids and products present in the ozonated mixture, was accomplished by gas chromatography mass spectroscopy (GC-MS). The GC-MS employed were an AutoSystem XL Gas Chromatograph, PerkinElmer and a TurboMass Mass Spectrometer (PerkinElmer) built in with a Zebron ZB-5MS GC capillary column. The software (PerkinElmer's Turbomass) that linked to a NIST database was used to identify the GC-MS chromatogram peaks. Several main compounds detected with high probability were reconfirmed by comparing their retention times to GC-MS standards bought from Sigma Aldrich (UK). All the standard chemicals chosen in the table were based on the highest probability suggested by the NIST database (Table 1). The settings highlighted below were used for the analyses: Auto sampler method: 2 μL injection volume; 2 μL pre-injection solvent washes; 6 μL post-injection solvent washes; Split mode: 20:1 μL; Temperature Program: 60–300 °C; Ramp 1: 5 to 300 °C min^{-1}; Carrier gas flow: constant 20 mL min^{-1} He gas.

2.6. Ozone generation and measurement

Ozone was generated by a Dryden Aqua ozone generator (corona discharge type) connected via silicone tubing to a glass diffuser type 4 with a pore size of 16 μm. These type 4 diffusers will produce an average size of 46 μm microbubbles at 0.1 L min^{-1} of air (Kokoo, 2015). First, a constant flow rate of ozone gas passes through a solution containing a certain concentration of potassium iodide. The products react with sodium thiosulphate ($Na_2S_2O_3$) 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:

$$\text{Ozone concentration}\left(\text{mg}\Big/\text{L}\right)=\frac{24\times Vs\times Ns}{V}$$

V is the volume of air bubbled (L), V_s is the volume of sodium thiosulfate (mL), and N_s is the normality of sodium thiosulfate (mg/me).

Table 2
Parameters restrictions of the CCF matrix.

Independent Variable		Level		
		−1	0	+1
A	Culture volume (mL)	30	65	100
B	Ozone concentration (mg L^{-1})	6	8	10
C	Time (min)	10	25	40

Table 3
Number of experiments suggested by the RSM software.

Run	Block	Factor 1 A:C.volume	Factor 2 B:O.conc	Factor 3 C:O.time	Response Chlorophyll
1	Block 1	65.00	8.00	25.00	
2	Block 1	65.00	8.00	25.00	
3	Block 1	30.00	10.00	10.00	
4	Block 1	65.00	8.00	25.00	
5	Block 1	100.00	6.00	40.00	
6	Block 1	100.00	6.00	10.00	
7	Block 1	65.00	8.00	25.00	
8	Block 1	100.00	8.00	25.00	
9	Block 1	65.00	8.00	10.00	
10	Block 1	65.00	6.00	25.00	
11	Block 1	30.00	6.00	40.00	
12	Block 1	30.00	10.00	40.00	
13	Block 1	30.00	6.00	10.00	
14	Block 1	65.00	10.00	25.00	
15	Block 1	100.00	10.00	40.00	
16	Block 1	65.00	8.00	25.00	
17	Block 1	100.00	10.00	10.00	
18	Block 1	65.00	8.00	40.00	
19	Block 1	65.00	8.00	25.00	
20	Block 1	30.00	8.00	25.00	

2.7. Statistical analysis using response surface methodology (RSM)

The process of optimization of chlorophyll reduction (algal cell disruption) by ozonation employs the Design Expert Version 6 software. This software uses statistical concepts to set up experiments. A central composite face-centred (CCF) setup was used to calculate the best possible values of culture volume, ozonation time and ozone concentration using a technique different from the conventional method. A CCF set up can help to increase the efficiency of an experiment. Restrictions were posted on the CCF matrix (Table 2). In this technique each factor has to be considered at 3 levels, the true limits are equivalent to the parameters related to the factors. For the purpose of this research the set-up must be able to predict the results produced by interaction among culture volume, ozone concentration and ozonation time.

The set-up of a CCF matrix which had a central point was prepared. The experiment was able to utilize the central composite design to decrease the chlorophyll content to the highest possible value since it can adhere to the quadratic surface which helps in the optimization procedure. A total of 20 experimental set ups were suggested (Table 3) in context of 3 factorial (2^3) designs and at the centre point there were 6 replications; the second order polynomial model saw the use of 6-star points. In Table 2 the highest and lowest values of culture volume,

Table 4
Optimization using statistical method.

Run	A	B	C	Chlorophyll (μg mL^{-1})
1	65	8	25	27.766
2	65	8	25	27.957
3	30	10	10	27.469
4	65	8	25	28.719
5	100	6	40	26.813
6	100	6	10	28.973
7	65	8	25	28.867
8	100	8	25	27.745
9	65	8	10	29.227
10	65	6	25	28.084
11	30	6	40	24.716
12	30	10	40	24.356
13	30	6	10	27.829
14	65	10	25	28.274
15	100	10	40	27.554
16	65	8	25	28.634
17	100	10	10	28.931
18	65	8	40	23.657
19	65	8	25	28.317
20	30	8	25	23.975

**A; Culture volume (mL), B; Ozone concentration (mg/L) and C; Ozonation time (min).

ozone concentration and time used in the research are presented. The lowest culture volume selected is 30 mL and the highest is 100 mL. The lowest ozone concentration selected is 6 mg L^{-1} and the highest ozone concentration is 10 mg L^{-1}. The minimum time selected is 10 min and the maximum time is 40 min. On the other hand, 20 cycles that had to be completed as predicted by the software are shown in Table 3.

3. Results and discussion

3.1. Optimization of algal cell disruption using response surface methodology

Design Expert Version 6.0.4 Software was employed to maximize algal cell disruption by analysing the reduction of chlorophyll content. According to Montgomery (2001), the Design Expert software uses statistics to formulate experiments. The range of ozone concentration, ozonation time and culture volume were optimized by using a central composite design (CCD). According to this methodology, each of the three elements must have three levels to set their true limit to allow the interaction and effects of those three factors to be assessed. The highest and lowest values of those factors were then determined. Traditional methods of optimization require fixing other variables (parameters) while changing one independent variable at a certain level. This single dimensional search is time consuming, painstaking and cannot reach a true optimum limit because it is unable to estimate interactions among experimental parameters. This statistical method is more practical, as it is developed from an experimental method that embraces interactions among the parameters (Bas and Boyaci, 2007). The use of a statistical methodology for product optimization has been widely employed. Wang and Lan (2011) reported on optimising the lipid production of green algae. Renita et al. (2014) reported optimising algal methyl esters using RSM. In this study, a statistical method that uses RSM was applied to optimize chlorophyll reduction by ozonation processes in a 0.2 L ozonation extraction bioreactor. The factors analysed are culture volume, ozone concentration and ozonation time.

3.2. Statistical analysis

Table 4 enumerates the various results produced in the central composite face centred (CCF) in terms of the chlorophyll content reduction of *D. salina*. Since the lowest culture volume level was 30 mL (15% minimum working volume), we decided to employ the central composite face centred design (CCF). During CCF, the value of alpha was kept constant at 1. Important variables were produced in the form of responses, and reduced models were prepared. The different properties of models were studied to predict the efficiency of the models.

Table 5
Analysis of variance (ANOVA) for the surface quadratic model.

Response	Model F-value	Prob > F	R^2 Value	Adjusted R^2 Value	Lack-of-fit	Standard Deviation
Chlorophyll reduction	4.75	0.0115	0.8104	0.6398	10.44	0.050

Table 6
Comparison of experimental, predicted value of chlorophyll reduction at the optimal levels predicted by RSM and comparison of reduction obtained from RSM and preliminary study.

Response	Response predicted	Response obtained (RSM)	Preliminary study	Improvement
Chlorophyll reduction	20.01%	18.58%	5.25%	254%

**Preliminary study was conducted in 0.2 L Ozonation bioreactor with 100 mL culture volume, 10 mg L^{-1} of ozone concentration and 40 min of ozonation.

A model considered as significant when the F-value is significant, the R^2 value is high, the standard deviation is low and the lack-of-fit F value is significant. Analysis of variance (ANOVA) was used to determine whether the model is statistically significant or not, as depicted in Table 5. The response (chlorophyll content) demonstrates that statistical significance can be accorded to the regression in which the probability value was > F (>0.0001). The F value obtained for the response is 4.75. With respect to all variables considered, the possibility of this Model F-value being produced due to chance is only 1.15%. Any values of "probability > F″ less than 5% is statistically significant. The optimum condition for the highest chlorophyll reduction suggested by the software was repeated three times. Comparison of experimentally predicted values of chlorophyll reduction at optimal levels predicted by RSM, and comparison of reduction obtained from RSM and the preliminary study is depicted in Table 6. The chlorophyll reduction predicted by the software for the optimum condition is 20.01%. On the other hand, only 18.58% chlorophyll reduction was gained after the optimum condition was repeated three times. The error could have occurred during the chlorophyll content analysis, but the difference (chlorophyll reduction) is not much. However, the reduction during the preliminary study is far too low (5.25%) and the improvement under optimum conditions is 254%. Thus, it is important to optimize the parameters to get the maximum effect, which in this case is the chlorophyll reduction.

3.3. Optimization using the desirability functions

The expected value for every response and variable was picked up from the menu during numerical optimization. The different milestones were the following: none, lying within the range, highest, lowest, target and for factors it would be to reach a precise value. Every variable must have a defined highest and lowest value. If the shape of the desirability function must be modified, then every milestone should be flagged with a weight. All of the milestones are considered in totality to obtain the total desirability function. Desirability measures the achievement of a milestone. 1 indicates that the milestone is reached; 0 indicates that the milestone is not reached. The aim of the program is to make the process more efficient. A random initial point is taken, and a steep slope is climbed to the highest value. Because the response surface has a curvature of more than 1, the highest value may be present, and the net sum is extrapolated to the desirability function (Malihe et al., 2008). With the

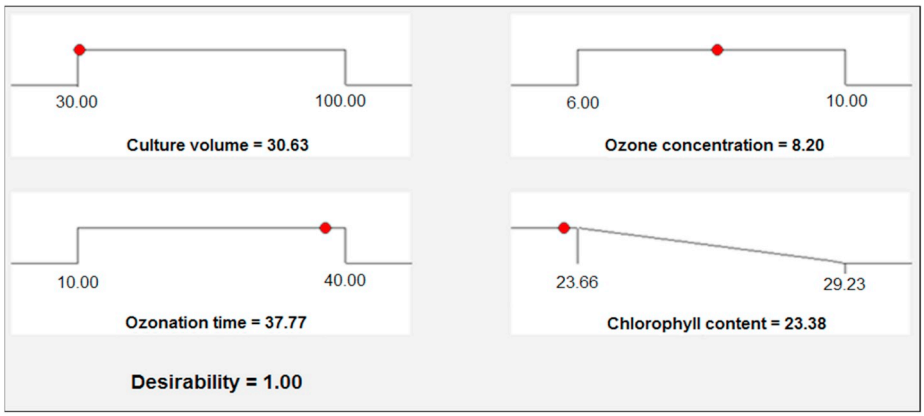

Fig. 2. Desirability ramp for numerical optimization of three factors, namely culture volume, ozone concentration and ozonation time.

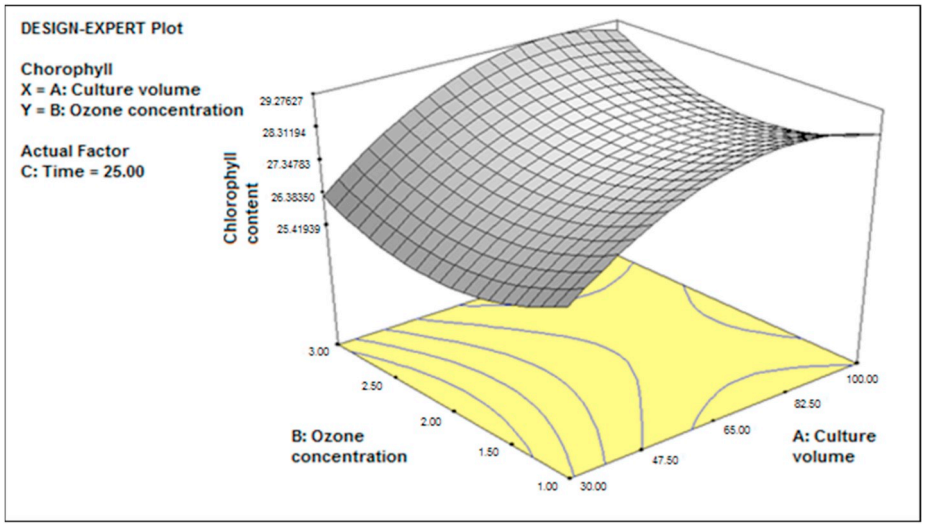

Fig. 3. RSM 3D graph.

help of numerical optimization, a point that helped in maximizing the desirability function was determined. All milestones are ranked in the context of other milestones (desirability ramp).

In Fig. 2, three parameters (culture volume, ozone concentration and ozonation time) are defined and depicted in the context of numerical optimization. The software produced six different solutions, which were used along with high desirability of 100%. The values at which the optimum local occurred are 30.63 mL of culture medium, 8.20 mg L^{-1} of ozone concentration and 37.7 min of ozonation time. The expected values for the highest chlorophyll reduction is 23.39 μg mL^{-1} (control = 29.23 μg mL^{-1}). The value of desirability at 1.00 shows that the function is representative of both the optimum and experimental set ups. The RSM 3D graph that shows the relationship of all the parameters is illustrated in Fig. 3. The lower the culture volume, the more it reduces the chlorophyll content due to highest surface contact. However, the highest ozone concentration (10 mg L^{-1}) does not produce the highest chlorophyll content reduction due to unstable (fluctuated) production of ozone. It has been reported that continuous corona discharges are not compatible for many applications due to a very low power. The voltage can be increased to raise the power level, unfortunately this leads into arcs. We believe that at the maximum volume of ozone generator, the device is tuned to prevent it from arcing. Stable ozone concentration is produced at the middle volume, which generates 8 mg L^{-1} of ozone concentration.

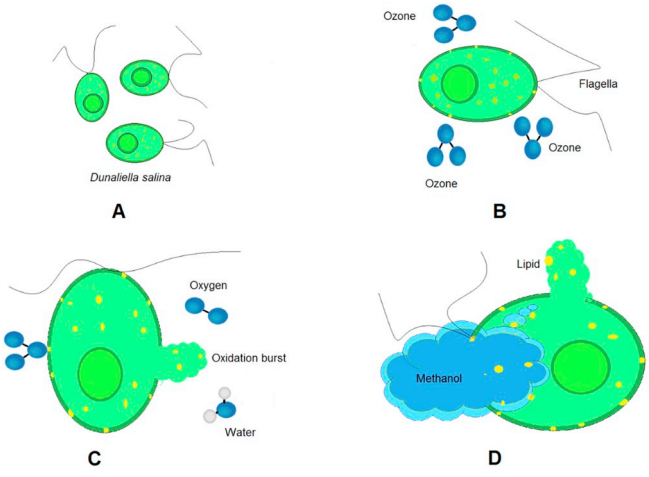

Fig. 4. Disruption mechanism of *Dunaliella salina* cells by ozone oxidation.

3.4. Microalgal lipid extraction and yields

The algal slurries were ozonated in a 0.2 L ozonation bioreactor to extract the lipids from the cells. Ozonation for 60 min has been reported to produced 3 main compounds (6,10,14-trimethylpentadecan-2-one;

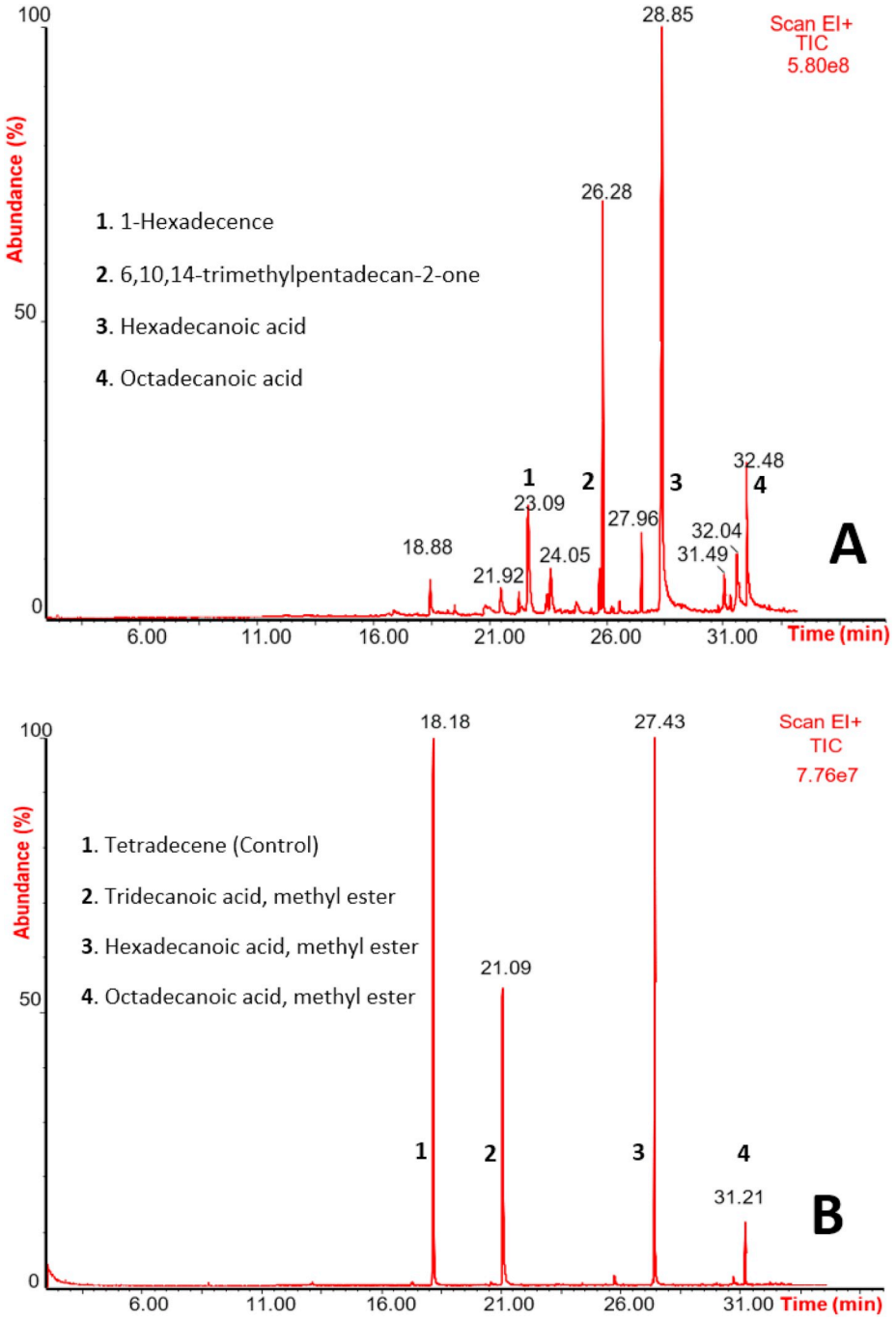

Fig. 5. GC-MS chromatograms of the A) 20 min ozonation sample, B) Further esterification of 20 min ozonation sample.

palmitic acid; stearic acid) (Kamaroddin et al., 2016). The obtained fatty acids include C16:0 and C18:1 which in agreement with the classification of *D. salina* in Chlorophyceae group. The classification of *D. salina* in Chlorophyceae group is well known and have been reported by many researchers (Assunção et al., 2012; Giordano et al., 2014). The possible disruption mechanism for *D. salina* cells by oxidation process is illustrated in Fig. 4. The cell membranes of *D. salina* cells are first attacked by ozone and unsaturated lipids and protein present in the membrane serve to be the prime target (Fig. 4 C). A reaction called oxidative burst occurred when the ozone molecule contacted with the cell membrane which creates a tiny hole resulting the cell to lose it shape. The presence of methanol will further disrupt and extract the internal lipids when in contact with the lipids inside of the *D. salina* cell (Fig. 4 D).

In this study, no catalysts (such as acid, alkali or enzymes) were used in the lipid extraction process to analyse the potential of direct transesterification by ozonolysis. Normally, in the production of fatty acid methyl esters (FAME), the transesterification reaction occurs only when the mono, di and tri varieties of acylglycerols are reacted with methanol in the presence of a catalyst (Xiao et al., 2009 and Kim et al., 2013). In the chemical industry, alkali catalysts are widely used for their higher reaction rates and conversion efficiency compared with acid catalysts (Huang et al., 2010). Based on comparison of the chromatogram peak with NIST database and methyl ester standards, no methyl ester was detected in the 20 min ozonation sample (Fig. 5 A). The ozonated samples contained of 4 main compounds (1-Hexadecence, 6,10,14-trimethylpentadecan-2-one; Hexadecanoic acid and Octadecanoic acid) were in agreement with the previous reported study (Kamaroddin et al., 2016). According to Kamaroddin (2017), there is a small amount of

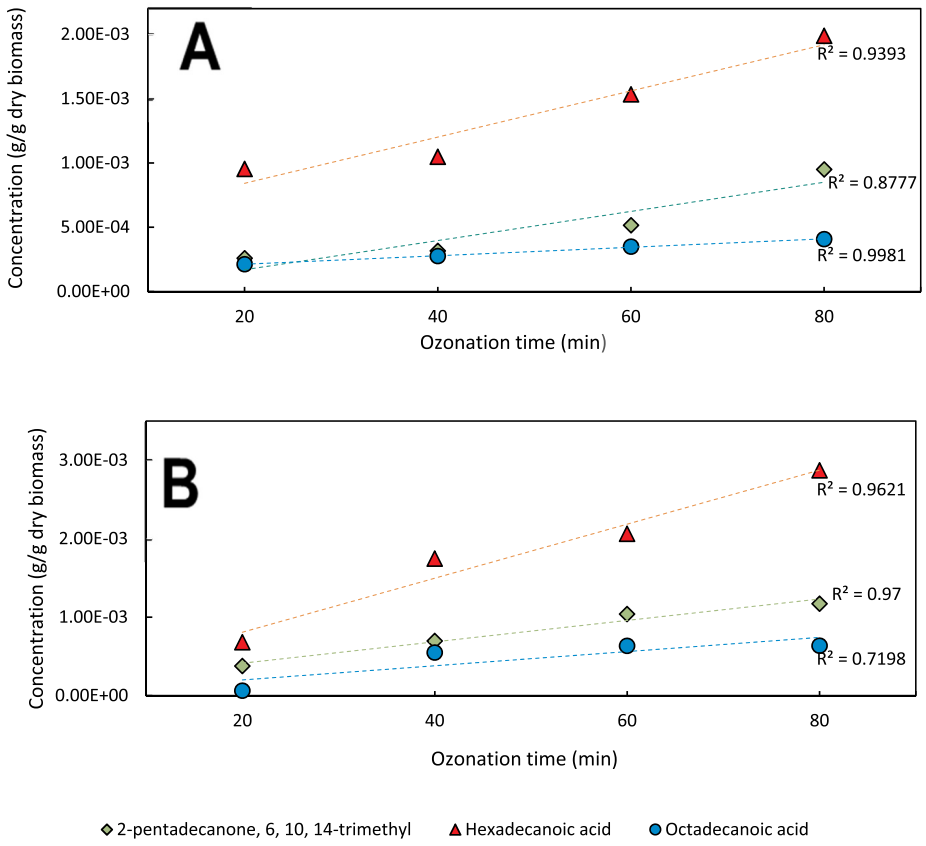

Fig. 6. Ozonation of microalgal slurry with methanol (1:5 v/v) in 0.1 L ozonation extraction bioreactor. A) Ozonation at 20° C without using fluidic oscillator, B) Ozonation at 20° C with fluidic oscillator.

methyl ester detected after ozonated for 20 min. However, the amount was too little as compared with the main products gained through the same process and was considered insignificant as it only conducted once. The algal lipid transesterification by using Method 2.4 of 20 min ozonated samples produced two ester products (hexadecanoic acid methyl ester and octadecanoic acid methyl ester), as expected (Fig. 5 B). Therefore, an optimization experiment was performed to study the temperature effect (20 °C and 60 °C) versus ozonation time. In addition, an experiment was also conducted to study the bubbles size effect with and without an oscillator during ozonation at the same time.

3.5. Optimization of algal lipid extraction

The study continued with the optimization of extraction processes to gain higher algal lipids. Two main factors were studied in this part: the temperature and the effect of smaller bubbles (generated by a fluidic oscillator). The experimental set up was the same as in the previous experiment (section 2.3); however, a 0.1 L Schott bottle was heated on a hot plate with the maintained and monitored temperature (Supplementary material 1). The experiments were divided into two parts: 1) ozonation for 80 min at 20° C with and without a fluidic oscillator, 2) ozonation for 80 min at 60° C with and without a fluidic oscillator. Due to an insufficient algal slurry, every experiment was conducted twice and with more diluted ratio of algal slurry over methanol than the previous experiments (1:5 v/v).

The ozonation at 20 °C increases the products (6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid) with prolongation of ozonation time (Fig. 6 A). Starting with 2.59×10^{-4}, 9.53×10^{-4} and 2.12×10^{-4} (g/g dry biomass) for 2-pentadecanone, 6, 10, 14-trimethyl; n-hexadecanoic acid and octadecanoic acid, respectively after 20 min of ozonation. It concludes with a maximum gained of 9.49×10^{-4}, 1.99×10^{-3} and 4.07×10^{-4} (g/g dry biomass) for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively after 80 min of ozonation. However, with the integration of a fluidic oscillator (previously proven to produce smaller bubbles), the concentration of the 3 products slightly improved (Fig. 6 B). The products that start with 3.78×10^{-4}, 6.82×10^{-4} and 6.31×10^{-5} (g/g dry biomass) after 20 min of ozonation end up with 1.17×10^{-3}, 2.87×10^{-3} and 6.37×10^{-4} (g/g dry biomass) for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively (after ozonation for 80 min). The product increments are 23.7%, 44.5% and 56.6% for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively as summarized in Table 7.

Table 7
Products improvement of ozonation lipid extraction at 20° C for 80 min.

Ozonation lipid extraction at (20 °C)	2-pentadecanone, 6, 10, 14-trimethyl (g/g dry biomass)	n-hexadecanoic acid (g/g dry biomass)	octadecanoic acid (g/g dry biomass)
Without FO	9.49×10^{-4}	1.99×10^{-3}	4.07×10^{-4}
With FO	1.17×10^{-3}	2.87×10^{-3}	6.37×10^{-4}
Improvement	23.7%	44.5%	56.6%

Further optimization of the algal lipid extraction process was carried out at 60 °C (Fig. 7). Generally, the ozonation at 60 °C increased the products yields with prolongation of ozonation time (almost similar trend with ozonation at 20 °C). Starting with 6.93×10^{-4}, 1.23×10^{-2} and 3.05×10^{-3} (g/g dry biomass) for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively after 20 min of ozonation, it ends with the maximum gained of 1.14×10^{-3}, 2.28

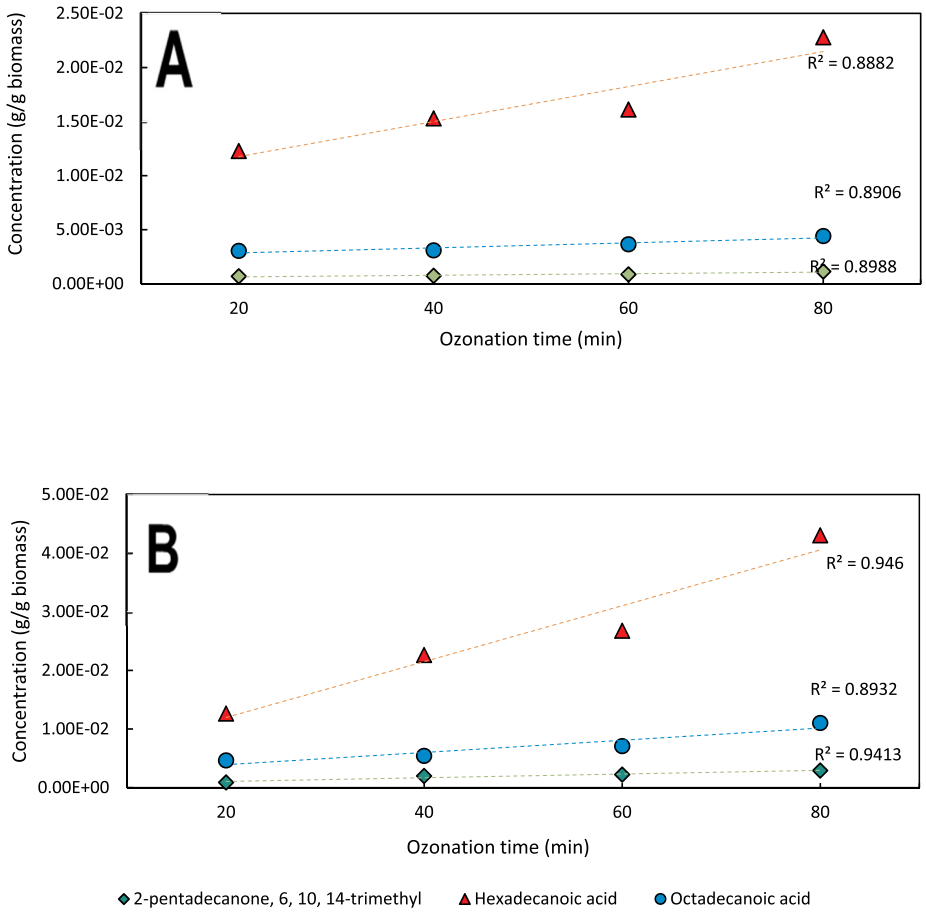

Fig. 7. Ozonation of microalgal slurry with methanol (1:2 v/v) in 0.2 L ozonation extraction bioreactor. A) Ozonation at 60° C without using fluidic oscillator, B) Ozonation at 60° C with fluidic oscillator.

Table 8
Products improvement of ozonation lipid extraction at 60° C for 80 min.

Ozonation lipid extraction (60 °C)	2-pentadecanone, 6, 10, 14-trimethyl (g/g dry biomass)	n-hexadecanoic acid (g/g dry biomass)	octadecanoic acid (g/g dry biomass)
Without FO	1.14×10^{-3}	2.28×10^{-2}	4.41×10^{-3}
With FO	2.91×10^{-3}	4.30×10^{-2}	1.10×10^{-2}
Improvement	156%	88.9%	150%

$\times 10^{-2}$ and 4.41×10^{-3} (g/g dry biomass) for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively after 80 min of ozonation (Fig. 7 A). However, with the integration of a fluidic oscillator, the concentrations of the 3 products were again slightly improved (Fig. 7 B). The products start with 8.79×10^{-4}, 1.26×10^{-2} and 4.63×10^{-3} (g/g dry biomass) after ozonation for 20 min and end with 2.91×10^{-3}, 4.30×10^{-2} and 1.10×10^{-2} (g/g dry biomass) for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively (after ozonation for 80 min). The products increased significantly with the use of the fluidic oscillator by around 156%, 88.9% and 150% for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively as summarized in Table 8.

Based on the experimental results, the advantages of using microbubbles are highlighted. Smaller sized bubbles require less time to dissolve gaseous compounds (ozone) than larger bubbles. Apart from that, the mass transfer also increases when the gas-flow rate increases. The integration of a fluidic oscillator significantly increased product formation compared to lipid extraction processes that do not use it. Even though pressure-assisted ozonation (PAO) methods have been reported to disrupt 80.3% of *Chlorella* cells and to produce 24% (w/w) of lipid

Table 9
Energy consumption of different microalgae extraction methods taken from recent studies.

Extraction Method	Microalgae	Energy consumption, (MJ kg^{-1} dry mass)	References
Bead mills	*Chlorella, Botryococcus, Scenedesmus,* (Laboratory, industrial)	504	(Lee et al., 2010)
Microwave	*Chlorella, Botryococcus, Scenedesmus,* (Laboratory)	420	(Lee et al., 2010)
Sonication	*Chlorococcum* sp., (Laboratory, industrial)	132	(Halim et al., 2012)
High pressure homogenization (HPH)	*Chlorococcum* sp. (Laboratory)	529	(Halim et al., 2012)
Hydrodynamic cavitation	*Saccharomyces cerevisiae* (Laboratory, industrial)	33	(Balasundaram and Pandit, 2001)
Ozone-rich Microbubbles	*Dunaliella salina* (Laboratory)	2.16	Recent study

yield (Huang et al., 2016), we still believe that our method is more energy efficient, because our method does not use a pressure mechanism. However, further study using our method should be conducted to make an apples-to-apples comparison.

3.6. Energy consumption and cost of lipid extraction by ozonation

In obtaining algal lipids, two major and expensive steps are involved: algal harvesting and lipid extraction (Lin and Hong, 2013). The energy demand for the centrifugation process is 90% of the energy gained in the production of the biodiesel; for press filtration the energy demand is approximately 79% of total energy and a further step that follows dewatering in the extraction of the solvent requires an extra 10% of the energy used in the production process (Brentner et al., 2011). Other routes such as ultrasonication and supercritical CO_2 also require large amounts of energy: about 110% and 66% respectively of the total biodiesel production energy (Brentner et al., 2011). From this explanation, it is clear that the whole of the energy budget in the production of biodiesel is consumed by the dewatering and lipid-extraction processes. Thus, it is crucial that the harvesting and extraction of lipids should be made more efficient in terms of the consumption of energy.

Energy consumption of different microalgae extraction methods taken from recent studies is summarized in Table 9. The method developed and utilised in this study uses microflotation (harvesting) followed by algal-ozone rupturing in methanol (disruption and extraction). According to Barathraj (2013) [43], normally 8 W of power is required by a good ozone generator (medium frequency, 800 Hz–1000 Hz) to produce 1 g of ozone. In this study 48 mg of ozone is required to extract 1 g of dry mass (within 60 min) which will be required 48 g of ozone to extract 1 kg of dry mass. Therefore, the energy consumption of the ozone generator is about 48 kW h which is equivalent to 172.8 MJ (1 kW h = 3.6 MJ). Based on an electricity price of £0.12 per kWh (UK Power, 2016), the cost of the ozonation extraction using ozone generator is about £5.76 per 1 kg of algal dry mass.

However, according to Lin and Hong (2013), commercially the amount of electricity required to generate ozone is in the range of 8–17 kW h kg^{-1} O_3. In this study, an ozone dose of approximately 48 g O_3 per kg dry algae was used to rupture algae and extract lipids. Thus, approximately 1.38–2.94 MJ kg^{-1} dry algae (384 W h to 816 W h) electrical energy would be required to generate ozone for cell disruption and lipid extraction. As such, the cost of energy for rupturing algae with ozone at 2.16 MJ kg^{-1} algae (average of 1.38 and 2.94 MJ kg^{-1} algae) is a small fraction of the energy that is used in the production of biodiesel (see Table 9). Additionally, the cost of algal lipid extraction using a commercial ozone generator is about £0.05 per 1 kg of algal dry mass which is based on an electricity price of £0.12 per kWh (UK Power, 2016).

According to Lin and Hong (2013), the production of biodiesel requires approximately 40 MJ kg^{-1} and the rupturing of algae requires approximately 6 MJ kg^{-1}. This is based on the assumption that 15% of the content of lipids can be converted to a similar amount of biodiesel. Unlike centrifugation and solvent extraction methods, which consume more than 90% of the energy, this method consumes only 36% (2.16/6) of the energy. Though the technique used in this study does not consider all the energy that a full-scale system may require, it is clear that employing this technique makes it possible to use less energy than that used in centrifugation and solvent extraction. However, this process has been tested on a non-cell wall microalgae with the assumption that the energy required to disrupt and extract lipids from *D. salina* cells is less than for a microalgae with a cell wall. Further studies should be conducted on microalgae containing cell walls such as *Chlorella* sp., *Chlorococcum* sp., *Botryococcus* sp. and *Scenedesmus* sp. in order to fairly compare the energy consumption and cost estimation for the cell disruption and algal lipid extraction for each microalgae.

4. Conclusion

A new ozone-rich microbubble bioreactor system for lipid extraction has been developed and tested. The concentration of products increased significantly to around 156%, 88.9% and 150% for 6,10,14-trimethylpentadecan-2-one; n-hexadecanoic acid and octadecanoic acid, respectively when the temperature was increased, and smaller bubbles were introduced during extraction. The energy usage for extracting *D. salina* lipid with ozone is lower compared to centrifugation and solvent extraction methods. Further studies should be carried out with microalgae containing cell walls and up scaling of the bioreactors to a larger volume is crucial in order to reduce the biodiesel production cost commercially.

Acknowledgements

M. Farizal Kamaroddin would like to thank the Ministry of Education Malaysia (MOE) for the financial support. We highly appreciate Simon Thorpe from the Faculty of Science Mass Spectrometry Centre for helping with the analysis of the GC–MS chromatograms.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101462.

References

Adam, F., Abert-vian, M., Peltier, G., Chemat, F., 2012. 'Bioresource Technology 'Solvent-free' ultrasound-assisted extraction of lipids from fresh microalgae cells: a green, clean and scalable process'. Bioresour. Technol. 114, 457–465.
Assunção, P., Jaén-Molina, R., Caujapé-Castells, J., de la Jara, A., Carmona, L., Freijanes, K., Mendoza, H., 2012. Molecular taxonomy of *Dunaliella* (Chlorophyceae), with a special focus on *D. salina*: ITS2 sequences revisited with an extensive geographical sampling. Aquat. Biosyst. 8 (1), 2.
Baratharaj, V., 2013. How to Evaluate and Select an Ozone Generator. OTSIL Fact Sheets.
Bas, D., Boyaci, I.H., 2007. Modeling and optimisation I: usability of response surface methodology. Food Eng. 78, 836–845.
Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37 (8), 911–917.
Brennan, L., Owende, P., 2010. Biofuels from microalgae- A review of technologies for production, processing, and extractions of biofuels and co-products. Renew. Sustain. Energy Rev. 14 (2), 557–577.
Brentner, L.B., Eckelman, M.J., Zimmerman, J.B., 2011. Combinatorial life cycle assessment to inform process design of industrial production of algal biodiesel. Environ. Sci. Technol. 45, 7060–7067.
Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25 (3), 294–306.
Giordano, M., Bargnesi, F., Mariani, P., Cryptogamie, S., 2014. Dunaliella salina (Chlorophyceae) affects the quality of NaCl crystals. Cryptogam. Algol. 35 (3), 285–302.
Griffiths, M.J., van Hille, R.P., Harrison, S.T.L., 2010. Selection of direct transesterification as the preferred method for assay of fatty acid content of microalgae. Lipids 45 (11), 1053–1060.
Halim, R., Harun, R., Danquah, M.K., Webley, P.A., 2012. Microalgal cell disruption for biofuel development. Appl. Energy 91 (1), 116–121.
Harun, R., Singh, M., Forde, G.M., Danquah, M.K., 2010. Bioprocess engineering of microalgae to produce a variety of consumer products. Renew. Sustain. Energy Rev. 14 (3), 1037–1047.
Huang, G., Chen, F., Wei, D., Zhang, X., Chen, G., 2010. Biodiesel production by microalgal biotechnology. Appl. Energy 87 (1), 38–46.
Huang, Y., Qin, S., Zhang, D., Li, L., Mu, Y., 2016. Evaluation of cell disruption of *Chlorella vulgaris* by pressure-assisted ozonation and ultrasonication. Energies 9, 173.
Kamaroddin, M.F., Hanotu, J., Gilmour, D.J., Zimmerman, W.B., 2016. In-situ disinfection and a new downstream processing scheme from algal harvesting to lipid extraction using ozone-rich microbubbles for biofuel production. Algal Res. 17, 217–226.
Kamaroddin, M.F., 2017. In-situ Disinfection and Algal Lipid Extraction Using Ozonation in Novel Microbubble Bioreactor for Biofuel Production. PhD thesis. University of Sheffield.
Kim, J., Yoo, G., Lee, H., Lim, J., Kim, K., Kim, C.W., Park, M.S., Yang, J., 2013. Methods of downstream processing for the production of biodiesel from microalgae. Biotechnol. Adv. 31 (6), 862–876.
Kokoo, R., 2015. Upgrading of Oleic Acid, Olive Oil, and Used Cooking Oil via Bubbling Ozonolysis. PhD thesis. University of Sheffield.
Lam, M.K., Lee, K.T., 2012. Immobilization as a feasible method to simplify the separation of microalgae from water for biodiesel production. Chem. Eng. 191 (15), 263–268.

Lee, J.Y., Yoo, C., Jun, S.Y., Ahn, C.Y., Oh, H.M., 2010. Comparison of several methods for effective lipid extraction from microalgae. Bioresour. Technol. 101, 75–77.
Lin, C.C., Hong, P.K.A., 2013. A new processing scheme from algae suspension to collected lipid using sand filtration and ozonation. Algal Res. 2 (4), 378–384.
Malihe, A., Habibollah, Y., Nader, B., Ali, A., Zinatizadeh, L., Farshid, G., Ali, D., Mazyar, S., 2008. Application of response surface methodology for optimization of lead biosorption in an aqueous solution by *Aspergillus niger*. J. Hazard Mater. 154, 694–702.
Mathimani, T., Pugazhendhi, A., 2019. Utilization of algae for biofuel, bio-products and bioremediation. Biocatal. Agric. Biotechnol. 17, 326–330.
Montgomery, D.C., 2001. Design and Analysis of Experiments. John Wiley and Sons, New York, pp. 427–450.
Mubarak, M., Shaija, a., Suchithra, T.V., 2015. A review on the extraction of lipid from microalgae for biodiesel production. Algal Res. 7, 117–123.
Prabakaran, P., Ravindran, A.D., 2011. A comparative study on effective cell disruption methods for lipid extraction from microalgae. Lett. Appl. Microbiol. 53 (2), 150–154.
Ranjan, A., Patil, C., Moholkar, V.S., 2010. Mechanistic assessment of microalgal lipid extraction. Ind. Eng. Chem. Res. 49 (6), 2979–2985, 17.
Renita, A.A., Sreedhar, N., Peter, D.M., 2014. Optimization of algal methyl esters using RSM and evaluation of biodiesel storage characteristics. Bioresour. Bioprocess. 1 (19), 1–9.
Salam, K.A., Velasquez-Orta, S.B., Harvey, A.P., 2016. A Sustainable Integrated in Situ Transesterification of Microalgae for Biodiesel Production and Associated Co−products − A Review', Renewable and Sustainable Energy Reviews, vol. 65. Elsevier, pp. 1179–1198.
Sivaramakrishnan, R., Incharoensakdi, A., 2019. Low power ultrasound treatment for the enhanced production of microalgae biomass and lipid content. Biocatal. Agric. Biotechnol. 20, 101230.
Surendhiran, D., Vijay, M., 2014. Effect of various pretreatment for extracting intracellular lipid from nannochloropsis oculata under nitrogen replete and depleted conditions. ISRN Chem. Eng. 1–9, 2014.
Tomiyasu, H., Fukutomi, H., Gordon, G., 1985. Kinetics and mechanism of ozone decomposition in basic aqueous solution. Inorg. Chem. 24 (19), 2962–2966.
UK Power, 2016. Gas and electricity tariff prices per kWh. Accessed on 30 November from. https://www.ukpower.co.uk/home_energy/tariffs-per-unit-kwh.
Wang, B., Lan, C.Q., 2011. Optimizing the lipid production of the green alga *Neochloris oleoabundans* using Box- Behnken design. Can. J. Chem. Eng. 89, 932–939, 2011.
Xiao, M., Mathew, S., Obbard, J.P., 2009. Biodiesel fuel production via transesterification of oils using lipase biocatalyst. Glob. Chang. Biol. Bioenergy 1 (2), 115–125.