Korean J. Chem. Eng., 34(4), 1100-1108 (2017)
DOI: 10.1007/s11814-016-0336-7

pISSN: 0256-1115
eISSN: 1975-7220

Application of high frequency ultrasound in different irradiation systems for photosynthesis pigment extraction from *Chlorella* microalgae

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(*Received 24 January 2016 • accepted 29 November 2016*)

Abstract−Microalgae are considered the biological drug factories of the future. To benefit from these microfactories, the intracellular metabolite of algae should be extracted. One of the most economically competitive methods is the ultrasound technique. This study was concerned with ultrasound-assisted extractions of useful substances from microalgae by comparing direct and indirect irradiation methods with respect to the extraction rate and yields. It is most likely that the direct and indirect irradiations had different irradiation powers. The systems were exposed to ultrasound wave (1.7 MHz) for 240 min. For each system, the changes of optical density, concentration and biovolume of *Chlorella* were estimated. In addition, the concentration of extracted chlorophylls (a, b and a+b), carotenoid and lipid were measured. The factors were studied after 30, 60, 120, 180 and 240 min of exposure to ultrasound irradiation. Both direct and indirect irradiation systems produced cavitation in the cell membrane, and they reduced the concentration and biovolume of the *Chlorella* cells. The amount of lipids and chlorophylls was greater in the direct irradiation as compared to the indirect one, and it caused more cell disruption. However, the extraction of the carotenoid was less effective because direct irradiation produced more transmitted power of ultrasound, resulting in degradation of carotenoid. The results and analysis presented in this research showed that selection of the best method of irradiation is an important step, and it depends on the biomaterials to be extracted.

Keywords: Irradiation, Microalgae, Ultrasound, Biomaterials, Carotenoid, *Chlorella*

INTRODUCTION

There are wide varieties of microalgal species [1,2]. They fix carbon dioxide through photosynthesis [2,3] and their growth rate is high so they can be cultured in a wide range of media [4]. Microalgae produce over 15,000 natural substances including enzyme, terpenes[1], carbohydrates [5], photosynthetic pigments such as chlorophylls and carotenoid [6], lipids and oils [7]. These advantages have made them unfailing resources for the isolation of natural components such as food additives, for health or biotechnological applications [1], cosmetics products [8] and biofuels[9]. Recently, the concept of using natural additives has attracted more attention due to changes in societal demand [8].

Nevertheless, several problems need to be resolved before microalgae-based substances can become economically achievable. One of the most difficult problems is to increase the extraction yield [10]. There are numerous reports in the literature on different extraction methods [11], such as homogenization, solvent extraction, ultrasonic-assisted extraction [2], mechanical pressing, milling, enzymatic extraction, microwave-assisted extraction [12], osmotic shock [1,7,10], hydrothermal acid treatment [13] and supercritical fluid extraction [9,14], each with its own advantages and drawbacks. For example, enzymatic treatment is limited by its high cost and low effectiveness. Physical methods are more cost effective; however, the rate of algal removal is low in most of them, with no possibility of scaling them up [15].

More recently, using ultrasound as a disintegration technique for algal cells has received broad attention in studies, as it provides an effective cost-efficient method for disrupting various algal species [6,16] without using any additive, while consuming less solvent [17] and saving energy [18]. Ultrasonic devices can be scaled up, operated continuously [19] and reach higher purity of the extracted metabolites [18,20]. Large-scale ultrasound extraction reactors in food [21-23] or chemical industries are designed in batch systems or in continuous mode in which a dry mass of about 10, 20 or 200 kg is extracted per hour [18].

However, there are reports on the degradation of some bioactive compounds after exposure to the ultrasound wave [24]. The other limitation of using high frequency ultrasound is the experiment temperature. Chematet al. showed that at higher temperatures, the ultrasound wave is less pronounced and the advantages are not exclusive [25]. In addition, dos Santos and Moreira (2015) compared different techniques for lipid extraction from *Chlorella vulgaris* biomass in terms of total extracted lipid and triglyceride. According to this study, the use of a mixture of organic solvents assisted by ultrasound is an efficient method in these extraction procedures [2]. Also, 30 kHz ultrasound waves were used to disrupt microalgal cell integrity and enhance lipid extraction efficiency [19].

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Ultrasound-assisted extraction has been also adopted to extract carotenoids, chlorophylls, and lipids from microalgae such as *Nannochloropsis* sp. and *Dunaliella salina* [6,7]. The ultrasound technique is a cost-effective, simple and fast technology for component extraction combined with different methods [26]. However, most of the reports on effects of ultrasound on lipid extraction yield are based on low frequency ultrasound (mostly 20-40 kHz) [27,28], while there are few studies on high frequency ultrasound. High-frequency ultrasound exhibits different advantages due to its short wavelength decreasing biological damages in the living tissues such as red blood cells [29]. High-frequency ultrasound waves cause physical forces and generate more free radicals as compared to low-frequency ultrasound because of more cavitation production. The released energy by cavitation bubble crashes make chemical bonds to break. Consequently, hydroxyl radials produced as water molecules are broken into hydrogen and oxygen atoms. This makes hydroxyl radials to be more remarkably produced, particularly through high-frequency ultrasound waves [30]. Wang and Yuan (2014) evaluated the effectiveness of high frequency (3.2 MHz) focused ultrasound in microalgal cell disruption. They reported that high frequency focused ultrasound was more energy efficient in the course of cell disruption [15].

However, ultrasound irradiation brings macro- and micro-algae cells into lysis, releasing their contents and thus reducing the required time for extraction. High-frequency ultrasound was used to harvest microalgae by separating liquids, and high intensity ultrasound causes enzymatic catalysis and heterogeneous activation of biomass reactions. In addition, the numbers of useful cavitations (per unit of time) with lower collapse temperature and smaller sizes increased in high frequency ultrasound irradiation. Efficiency of cavitation and releasing energy per bubble are high at higher frequencies [31].

Many researches have compared extraction of biomaterials from microalgae biomass through different ultrasound frequencies using other factors such as time fraction and different solvents. However, comparison between ultrasound-assisted extractions at a constant frequency of ultrasound wave with different methods of irradiation is yet to be reported. Generated acoustic field in each ultrasound transducer is different from that of the others. This can influence the optimal value of the experimental results [32]. Therefore, the main issue to be addressed in the present research is the effect of irradiation methods on ultrasound-mediated extraction of photosynthetic pigments from green microalgae, *Chlorella* sp. through a high frequency (1.7 MHz) transducer. For this purpose, two systems were designed: the direct and indirect exposure to ultrasound irradiation systems. Other factors such as sample volume, inoculated microalgae, temperature, and light were the same in both systems.

MATERIALS AND METHODS

1. Experimental Setup

When extracting biomaterials using ultrasound, the selection of ultrasonic parameters, ultrasonic reactor type, frequency, temperature, aeration of reactors and type of solvent determines the level and distribution of energy intensity in the system, influencing efficiency of ultrasound irradiation and reliability of the results [31,33]. Depending on the research objectives, the selection of ultrasonic reactors and optimization of parameters can be quite different. In the present study, to obtain comparable results, the procedure was carried out on both systems under the same microalgae culture conditions, including sample volume, microalga population and medium. Based on the experimental objectives and since these factors can affect acoustic power of ultrasound, different ultrasonic reactor types were designed to reduce the range of power of ultrasound with different irradiation methods.

To study the bioeffect of high frequency ultrasound on *Chlorella* sp., a set of 1.7 MHz piezoelectric transducers (Model ANN-2517GRL, AnnonPiezo Technology Co. Ltd., China) were used. Also, the following protocols were employed: i) direct irradiation of high frequency ultrasound, ii) indirect irradiation of high frequency ultrasound through water bath, and iii) *Chlorella* sp. culture without exposure to the ultrasound wave, as the control. A schematic layout of transducers is shown in Fig. 1.

In the literature, power ratings of acoustic levels describe the systems of experiments. Sonochemical reactors are specified through their power-rating values. However, these power-rating values are not practically consistent because there are always some energy losses through ultrasound wave transformations [30]. The acoustic power (P) of the transducers was calculated using standard calorimetric method to measure the increase in temperature (ΔT) of 500 ml of culture over irradiation time (Δt) using the following equation [30,34-36]:

$$P = \frac{m \times C_p \Delta T}{t}, \quad (1)$$

where m is the mass of liquid (kg), C_p is the specific heat capacity (J/kg K), ΔT is the temperature rise of water (K), and E is electric energy supplied (W) for the time interval of t (s).

This method was used in the studies on microalgae and other microorganisms [30,35].

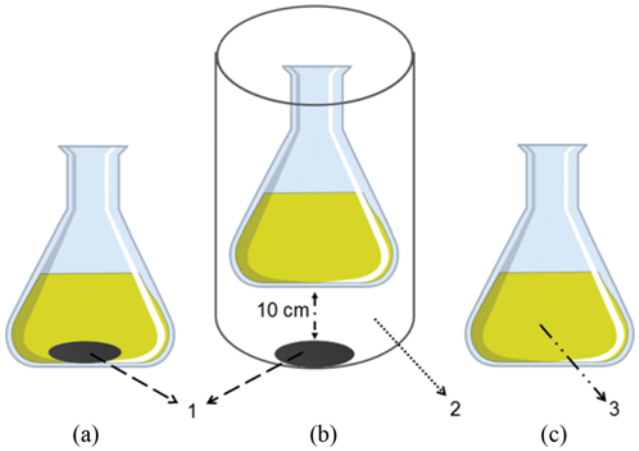

Fig. 1. Schematic design of experimental protocols (n=3), (a) direct exposure to ultrasound irridation, (b) indirect exposure to ultrasound irridation, (c) control (without exposure to the ultrasound wave).
1. Piezoelectric transducers 3. Suspension of *Chlorella* sp.
2. Water

The power levels are proportional to the volume of experimental sample. Therefore, the ultrasound power density was measured as follows [37]:

$$\text{Power density} = \frac{P}{V} \quad (2)$$

where P is the calorimetry power (W) and V (ml) is the sample volume.

Chlorella sp. was cultured in BG11 medium, consisting of 1.5 g, 0.04 g, 0.075 g, 0.036 g, 0.006 g, 0.006 g, 0.001 g, 0.02 g, 1.0 ml and 1.0 L of $NaNO_3$, KH_2PO_4, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, citric acid, ferric ammonium citrate, EDTA disodium salt, $NaCO_3$, trace metal mix and distilled water, respectively. A uniform suspension (Bausch and Lomb70, $OD_{680\,nm}$~0.221±0.002 °A, spectrophotometer) was inoculated into the ultrasound exposure bioreactor system [19]. An Erlenmeyer flask (1,000 L) was used as the photobioreactor (Fig. 1). The experiments were carried out at room temperature (25.4± 2.6) for 240 min.

They were illuminated with cool white fluorescent lights from two metal Halide lamps at 50 cm from the surface of the photobioreactor. Such arrangement gave an average light intensity of 75-80 μmol photons $m^{-2}s^{-1}$.

Efficiency of mixing through ultrasound waves has been investigated in different studies mainly on low frequency reactors. High frequency reactors (at frequencies higher than 1 MHz), produce small bubbles and contribute to the production of acoustic streams in the liquid as well as mixing effect. High frequency ultrasound makes micro-streams and convective flows to be generated simultaneously. Thus, more effective macro- and micro-mixings are possible to obtain [38]. Therefore, other mixing methods were not used in this research.

1-1. The Effect of Exposure Time

The reports showed that different factors such as concentration of inoculate microalgae, biosubstance extractability yield, temperature of experiment, the type of extraction solution and its volume affect the time of exposure to the ultrasound wave.

Bigelow et al. studied the application of high-intensity focused ultrasound on lysis of *Chlamydomonas reinhardtii* as a function of exposure time; they showed that longer exposures of ultrasound wave completely degraded the cells, releasing almost all the intracellular materials of microalgae into the supernatant. Also, they studied changes of the concentration of microalgae and extraction yield by changing the exposure time [39]. Ometto et al. showed the lower temperature by using longer treatment times to increase extraction yield [48]. Araujo et al. studied the optimal method of ultrasound assistant extraction. The time exposure was different in every method [40].

To evaluate the effect of exposure time, various physiological parameters of *Chlorella* sp. such as microscopic counts of species populations, photosynthetic pigments (Chlorophyll a, b, a+b and carotenoid) and lipid extraction, were measured after 30, 60, 120, 180, and 240 min of exposure to ultrasound, in both systems.

2. Relative Change of Cell Concentration

Cell concentration values were estimated through microscope counting of species populations. Neobar lam was used to estimate the number of cells per unit volume. In addition, the absorbance of microalgae was measured at a wavelength of 680 nm. The relative change in cell concentration (RCCC) is defined as follows [15]:

$$\text{RCCC} = \frac{\text{cell concentration of the treatment} - \text{cell concentration of the control}}{\text{cell concentration of the control}} \times 100 \quad (3)$$

where N_0 is the initial concentration of microalgae (number per unit volume) and N is the final concentration. The measurements were carried out during a period of 240 min.

3. Biovolume Measurement

The biovolume of algae cells was estimated as a measure of relative algal biomass. Biovolume was measured based on geometric shapes. *Chlorella* cells are spherical, so the biovolume was estimated according to the volume of a sphere. The radius of *Chlorella* cells was measured with Dino Capture 2.0 with an Olympus light microscope (40×).

4. Chlorophyll and Carotenoid Determination

Cells were collected by centrifuging samples at 13,000 rpm for 10 min. There was no relaxing time between US treatment and solvent extraction. The pigments were extracted with a 90% methanol solvent (solvent : sample ratio of 1 : 1%v/v). Once centrifuged, the solvent (sediment) was introduced into algae cells before they were mixed through a vortex. Cell debris was removed by centrifugation at 13,000 rpm for 10 min. The absorbance of the solvent extract was measured at 665 and 650 nm wavelengths. Solvent was used as blank and the extracted chlorophyll was measured by using the following equations [41]:

For 90% methanol (mg/*l*):

$$\text{Chlorophyll a} = (16.5 \times A_{665}) - (8.3 \times A_{650}), \quad (4)$$

$$\text{Chlorophyll b} = (33.8 \times A_{650}) - (13.5 \times A_{665}), \quad (5)$$

$$\text{Chlorophyll a+b} = (4.0 \times A_{665}) + (25.5 \times A_{650}). \quad (6)$$

The concentration of total carotenoids was calculated by using the following equation [6]:

$$C_{total\ carotenoids\ (x+c)} = \frac{1000_{A470} - 1.63C_a - 104.96C_b}{221}, \quad (7)$$

where A_{470} is the absorbance at 470 nm, C_a and C_b are the concentrations of chlorophyll a and b, respectively.

Pigments and lipids were extracted from the minimum volume of microalgae culture in each sample (30, 60, 120 and 240 min). The feed volume was 500 ml, while the extract volume for pigment extraction and OD was 3 ml. Therefore, at the end of the experiment, only 12 ml of the feed was consumed, while a residual amount of about 488 ml was obtained. The mass balance (feed/extract/residue) for pigments extraction and OD was 500/12/488.

5. Ultrasound-assisted Lipid Extraction

Ultrasound waves propagated inside a fluid as a periodic sound pressure are defined as follows [42]:

$$P_S = P_A \cos\left[2\pi f\left(t + \frac{y}{c}\right)\right], \quad (8)$$

where, c is the sound speed in the fluid, f is the wave frequency, and y and t are space index and time, respectively. In addition, P_A is the acoustic pressure calculated according to the following equation:

$$P_A = \sqrt{2\rho_L I_{US} C}, \quad (9)$$

in which I_{US} and ρ_L are defined as the ultrasound intensity and the liquid density, respectively [43].

Due to ultrasound wave propagation, a reduction in local pressure to below vapor pressure of local liquid was likely to occur, leading to formation of cavitation bubbles. Bubbles move until they implode, making shock waves, by a phenomenon called transient cavitation [42,44]. Cavitation bubble sizes have an inverse relationship with the frequency: high frequency ultrasound propagation produces smaller cavitation bubbles with weaker implosions as compared to low frequency ultrasound [43]. However, at the same power, more cavitation bubbles can be produced by higher frequency waves as compared to lower frequency ones. In the present study, high frequency (1.7 MHz) ultrasound waves were employed to enhance lipid extraction from microalgae.

There are various methods of lipid extraction from microalgae. In this research, lipids were extracted through modified wet biomass extraction method. A solution of methanol: chloroform (1 : 2%v/v) was used as the extract solvent. There was no relaxing time between the US treatment and solvent extraction. A volume of 9 ml of the solvent (methanol : chloroform) was added to 9 cc of microalgae culture. This mixture was shaken gently for 3 min at room temperature before being put into a decanter. When a two-phase mixture was established, the organic phase was carefully collected while the solvent was evaporated with a rotary evaporator at 60 °C. The lipid fraction was dried to constant weight in an oven with air circulation at 30 °C [45]. In both systems, lipid extraction yield was measured after 30, 60, 120, 180 and 240 min exposure to the ultrasound wave, to evaluate the effect of exposure time. Time 0′ represents the initial content of pigments and lipids in the culture.

In each run, a 9 ml sample was used to extract lipids, so that at the end of the experiments, 464 ml of feed was still intact. Therefore, the mass balance (feed/extract/residue) for lipid extraction was 500/36/464.

6. Statistical Analyses

The effect of ultrasound waves on microalgae was studied with three replications in all the systems. The results were reported as mean±standard error. ANOVA analysis of SPSS version 16.00 and Duncan test were used to study the significant mean differences.

RESULTS AND DISCUSSION

1. Energy Input Study

The actual energy dissipated in both systems was determined by recording algal suspension temperature. Acoustic power (energy input) was estimated to be 7.5 and 2.5 W in direct and indirect systems, respectively. Considering the experimental sample volume of 0.5 L in both systems, power density was calculated (Fig. 2).

2. Effect of Ultrasound on Algae Removal

The effect of ultrasound on algae removal was evaluated in terms of cell concentration value (cells/L). Optical density at 680 nm ($OD_{680\,nm}$) was estimated as an indication of the extent of cell disruption. The biovolume change is another direct indication of the algae cell removal.

3. Cell Concentration Changes

The changes in cell concentration within all the systems are summarized in Fig. 2. According to the results, ultrasound irradiation affected cell concentration with a significant reduction (*p value* 0.000) as compared to the control in both systems. This reduction was caused by the mechanical damage in the structures of algae cells due to ultrasound cavitation [46].

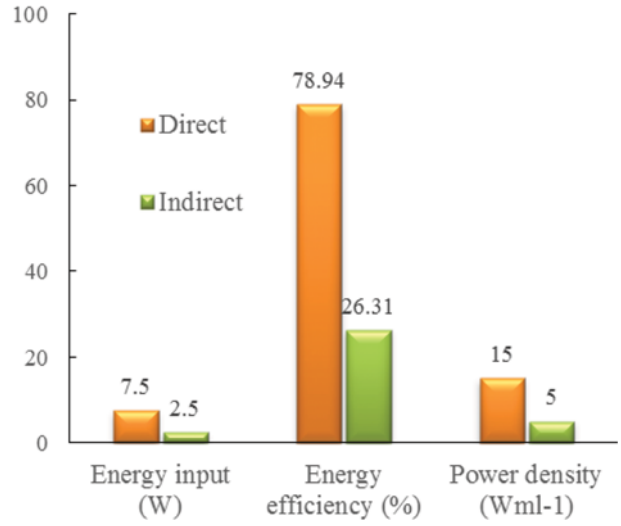

Fig. 2. Energy input and power density of direct and indirect system.

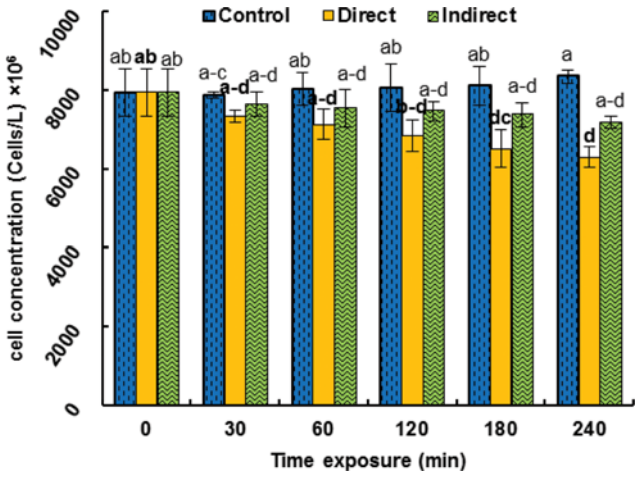

Fig. 3. The effect of ultrasound on cell concentration (Cells/L) ×10⁶.

As shown in Fig. 3, the cell concentration of the control did not change significantly during the experiments. There was no significant difference in cell disruption between the direct and indirect systems up to 120 min. The best removal effect was achieved in the direct systems. Direct exposure to the ultrasound irradiation caused 20.7% cell disruption after 240 min of laboratory-scale experiment. The energy input of the direct system was three-times higher than that of the indirect one. Higher power of ultrasound enhances the production of cavitation [19]. The cavitation mechanism is related to the species of algae due to their different structures [47]. For example, the ultrasound wave causes cavitation in intracellular gas-vacuoles of cyanobacteria [29]. The results of this experiment are in good agreement with numerous reports on the use of different frequencies of ultrasound. For example, Wang et al. (2014) reported that algae cells could be disintegrated when the

input energy is sufficient. Although the mechanical energy of cavitation is less at high frequencies of ultrasonic waves, a larger proportion of free radicals are generated due to ultrasonic water degradation by ultrasound irradiation. The radicals attack cell membranes, thus leading to lysis of cells. They studied the ultrasound effect on disruption of *Nannochloropsis oculata* microalgae at ultrasound wave frequencies of 3.2 MHz and 20 kHz as well as input energy values of 40 and 100 W, respectively [15]. Since different frequencies of ultrasound were used in this study, the results are not comparable when finding the best yield for use in the industries.

During the experiment, the temperature increased to 44 °C. To study the effect of temperature on algae growth, *Chlorella* sp. was cultured in BG11 medium at 44 °C for 24 h. The results of cell concentration and $OD_{680\,nm}$ showed a growth in the algae culture. Therefore, the reduction of cell concentration in ultrasound-exposed system was due to ultrasound wave irradiation.

4. Changes in the Measured Optical Density

The OD measurements at 680 nm followed the same trend as the cell concentration. The reduction was significant in ultrasound irradiation systems as compared to the control (*p value* of 0.000) with significant differences between direct and indirect exposure systems (*p value* of 0.002). Fig. 4 shows that the maximum removal (23%) was achieved at 240 min in the direct systems. Therefore, $OD_{680\,nm}$ could be used as a surrogate parameter for cell disintegration.

The influence of 30 kHz ultrasound frequency on microalgal cell integrity was studied by Keris-Sen et al. (2014) in wastewater and BG11 mediums [19]. They revealed that the application of OD measurements relied on the structure of algal cells in distinctive cultures. Biovolume of *Chlorella* sp. cells was estimated to study the differences in removal percentage in terms of cell concentration and $OD_{680\,nm}$.

5. Biovolume-based Growth Changes

The impact of ultrasound on biovolume-based growth is shown in Fig. 5, where biovolume in the ultrasound-exposed system was significantly lower as compared to the control (*p value* of 0.000).

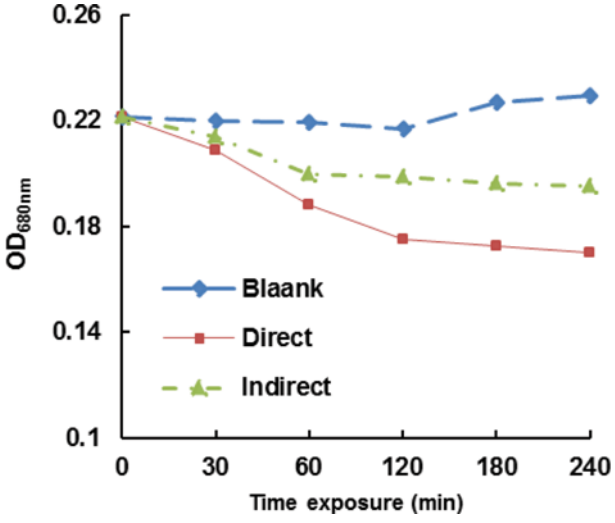

Fig. 4. Measured optical densites at 680 nm during the experimental times.

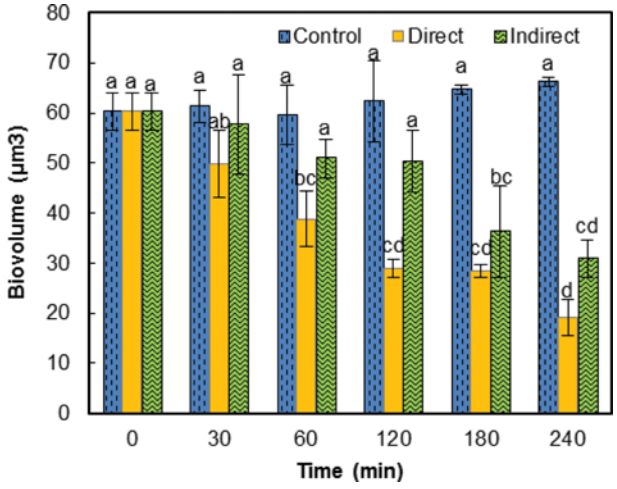

Fig. 5. Biovolume changes in the direct and indirect systems during 240 minutes.

As shown in Fig. 5, the small size of *Chlorella* sp. is an indication of cell damage and cavitation, making intracellular matrix to leak out, hence enhancing bio-substance extraction from microalgae cells [19]. Therefore, an increase is expected in the extraction yield of hydrophilic and hydrophobic metabolites with decreasing biovolume. These results show the differences between reduction percentages of cells concentrations and $OD_{680\,nm}$. During the experiment, the biovolume of the control did not change efficiently (*p value*<0.05) as shown in Fig. 5. However, the biovolume of the directly exposed system changed significantly in comparison with that of the control during the experiment. The biovolume changed significantly in the indirect system after 120 min (*p value*>0.05 for up to 120 min).

The changes in cell concentration, $OD_{680\,nm}$ and biovolume-based growth implied that direct exposure to ultrasound wave was more effective than the indirect system of exposure in disintegration of *Chlorella* sp. cells. Because the frequency was the same (1.7 MHz) in both systems, the growth rate was found to be different due to different values of energy input efficiency (Fig. 6). These results show that energy input has more impact on the effectiveness of algal inhibition growth and cell disintegration than the frequency, which is in agreement with Heng et al's (2009) results [48]. Therefore, the energy input should be considered when using ultrasound for algal disruption in such applications. The energy input depends on algae species and their physical characteristics, which include shape, size, structure [15] as well as volume of the sample [49]. There are numerous researches on ultrasound application for algae removal at different frequencies and energy input values; however, many of them used low frequency ones [50]. Application of a high frequency (1.7 MHz) ultrasound for *Spirulina plantensis* removal with a power intensity of 0.6 WL^{-1} for 9 min caused inhibition of growth [tang]. In other studies, a power intensity of 0.07 WL^{-1} for 5 min was associated with a reduction of about 50% of *Spirulina* cells [29,47].

6. Effect of Ultrasound on the Photosynthetic Pigments Extraction of *Chlorella* sp.

To study the impact of ultrasound on the *Chlorella* sp. photosynthetic pigments extraction, *chlorophyll a*, *b*, *a+b* and carotenoid were

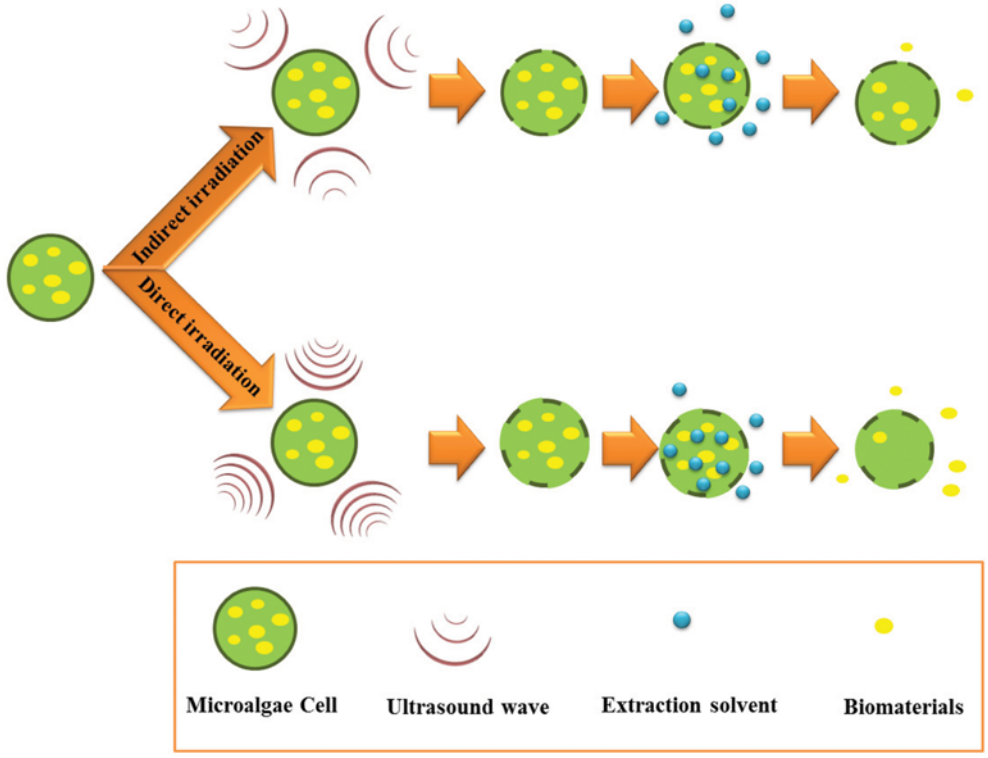

Fig. 6. Schematic diagram of iiradiation effect on microalgae cells in the direct and indirect systems.

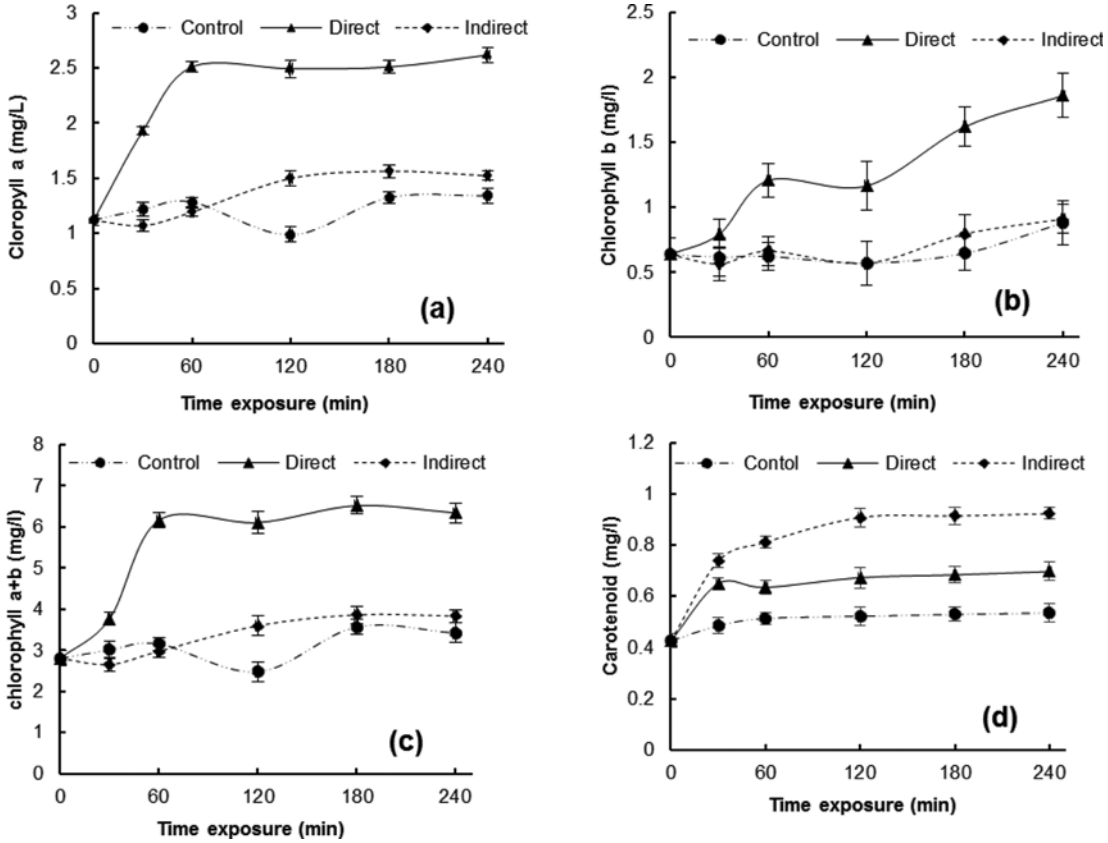

Fig. 7. Effect of ultrasound wave on the amount of photosynthetic pigments extracted from *Chlorella* sp. (a) chlorophyll a (b) chlorophyll b (c) chlorophyll a+b (d) carotenoid.

studied.

6-1. Effect of Ultrasound on the Amount of *Chlorophyll a* Extracted

Fig. 7(a) shows the profile of *chlorophyll a* for the extract obtained by high frequency ultrasound technique. The results show that chlorophyll extraction was increased significantly (*p value* 0.00<0.05) through direct exposure to high frequency ultrasound. The extraction yield in the direct system reached 2.61 mg/L, while it had a maximum of 1.52 mg/L in the indirect system.

6-2. Effect of Ultrasound on the Amount of *Chlorophyll b* Extracted

As shown in Fig. 7(b), the amount of *chlorophyll b* extracted from *Chlorella* sp. increased when the system underwent high frequency ultrasound treatments. Although direct exposure made the extraction process to be significantly improved in comparison with the indirect exposure and control systems (*p values* of 0.007 and 0.005<0.05, respectively), there was no significant difference between indirect systems and the control (*p value* 0.87>0.05).

6-3. Effect of Ultrasound on Chlorophyll a+b Extraction

As shown in Fig. 7(c), the effects of ultrasound on total chlorophyll extracted from *Chlorella* sp. in the direct system caused a significant change in the yield value (from 2.803 to 6.343 mg/L). Similar to *chlorophyll a* and *b*, a remarkable increase was evident in total chlorophyll yield. Moreover, a significant difference (*p value* of 0.002<0.05) was observed between the direct system and the control, in this regard. The direct extraction curve of *chlorophyll a* and *a+b* increased logarithmically up to 60 min after it exhibited an approximately stationary state.

The results show that evolution of all the three chlorophyll extraction curves followed the same trend. These results are consistent with those obtained in a previous study by Macías-Sánchez and Mantell (2009), in which a discussion was presented on the extraction of carotenoids and *chlorophyll a* from *Dunaliella salina* through the ultrasound technique. In addition, the results obtained are in agreement with those of Kong and Liu (2012) and Wang and Yuan (2014), showing that the enhancement observed in the yield of pigment extraction from the algae is the result of the increase in the contact area between the microalga and solvent.

Since chlorophylls are sensitive to high temperatures, the extraction process through the solvent was carried out in a dark room at ice temperature to prevent the degradation of chlorophylls. Although the temperature increased in the current experiments, this study highlighted the potential application of direct irradiation of a 1.7 MHz ultrasound for yield enhancement of chlorophyll, the contribution of changes as a function of exposure time. This result showed that photosystem II efficiency was not distorted by ultrasound irradiation [51]. Based on the kinetic extraction study, it was deduced that direct exposure to an ultrasound wave is more effective, since the transmitted power is higher than that in the indirect system. Based on the results obtained, the exposure duration of 60 min may serve as an appropriate starting point for future researches.

6-4. Effect of Ultrasound Irradiation on Carotenoid Extraction

Changes in the amount of carotenoid extracted are presented in Fig. 7(d), where the carotenoid extraction curve for indirect ultrasound irradiation is above all the other curves. Indirect ultrasound irradiations remarkably enhanced the extraction yield (*p value*<0.05), ranging from 76 to 119%. Despite high extraction yields observed for the chlorophylls in the direct exposure system, extraction of carotenoid was less effective in the directly exposed system as compared to the indirect one with higher yield values for longer experimental times. Based on the results obtained, ultrasonic-assisted extraction was adopted to extract carotenoid from *Chlorella* sp. [26], because ultrasound cavitation facilitates the passage of solvent through microalgae cell membranes [6]. However, cavitation may accelerate or trigger chemical reactions within the extraction media. Thus, it results in degradation of carotenoid due to its polyisoprenoid structure consisting of a long conjugated chain of carbon-carbon double bonds [52].

Although the carotenoid yield increased remarkably in the direct system, it was still less than that in the indirect one. It appeared to be caused by temperature enhancement in the indirect irradiation. It can be explained by the study of Macías-Sánchez et al. (2009), which reported ultrasound-assisted extraction of carotenoids and *chlorophyll a* from *D. salina* in different temperatures, pressures and different solvents [6]. They found that these factors can change the extraction yield. The highest yields in carotenoid and chlorophyll extraction were obtained at a temperature and pressure of 60 °C and 400 bars, respectively. Therefore, an increase in temperature did not decrease the carotenoid extraction yield, that is, the greater the power of ultrasound, the greater the degradation of carotenoid.

6-5. Effect of Ultrasound Irradiation on Carotenoid/Chlorophyll Ratio

The carotenoid/chlorophyll ratio (Car/Chlo) was estimated as a selective efficiency parameter for the extraction of carotenoid. Car/Chlo ratio presented in Fig. 8 shows higher value in the indirect systems as compared to the indirect ones. This means that Car/Chlo ratio increases with decreasing power in the indirect system. One can conclude that carotenoid extraction had its maximum efficiency when low transmittance power irradiation was applied, which is similar to the findings of Macías-Sánchez and Mantell (2009) [6].

7. Effect of Ultrasound on the Amount of Extracted Lipid

The amounts of extracted lipid (g/L) are shown in Fig. 9. The ultrasound application significantly contributed to lipid extraction for both the direct and indirect systems.

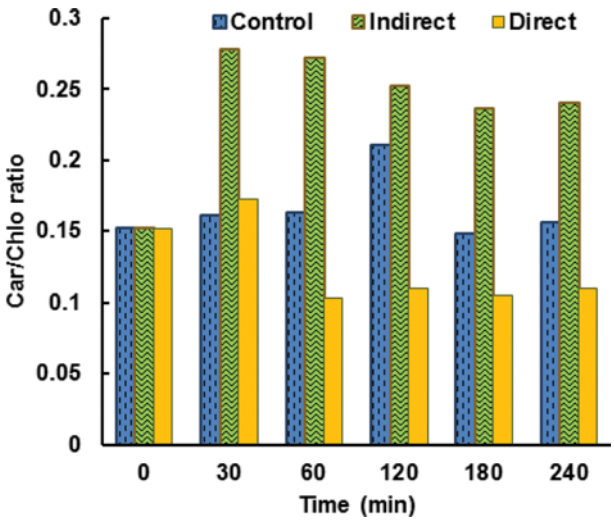

Fig. 8. The carotenoids/chlorophylls ratio.

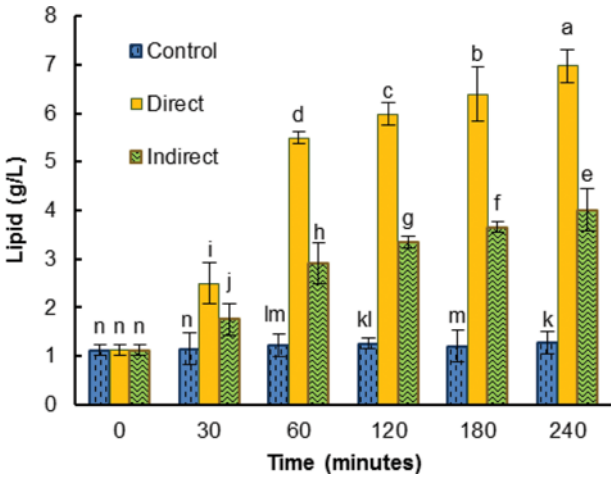

Fig. 9. The amount of extracted lipids from *Chlorella* sp. using chloroform/methanol (2 : 1%v/v) under ultrasound treatment.

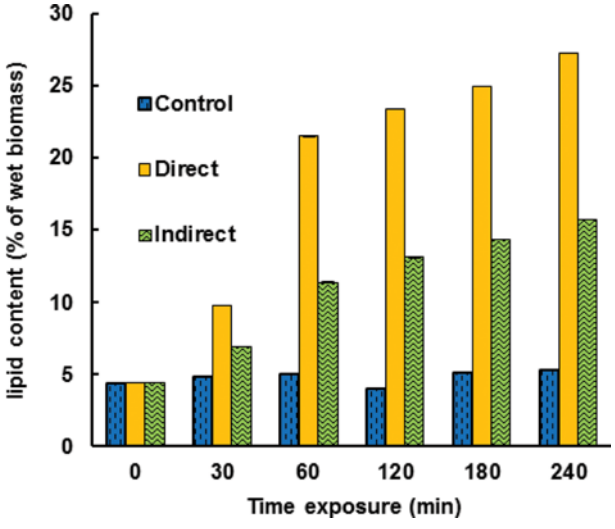

Fig. 10. The change in lipid content (% of biomass, wet weight) of *Chlorella* sp. with chloroform/methanol (2 : 1) with ultrasound in both direct and indirect systems and control.

Lipid extraction yields are shown in Fig. 10, as percentages of wet biomass. The results show that high frequency ultrasound enhanced lipid extraction yield by 6- and 4-fold in the direct and indirect systems, respectively. The two sets of experiments revealed that ultrasound significantly contributed to lipid extraction. The maximum extracted lipid (27.3% wt) was obtained by using co-assistance of the solvent as well as the direct exposure to ultrasound for 240 min. A relatively lower lipid yield (4.4-5.3% wt) was obtained in the control system through solvent extraction. These results indicated that direct sonication promoted efficiency in capturing lipids released from wet biomass due to higher induced power for the *Chlorella* sp. cells. This is in agreement with earlier studies of [18,53,54].

Intracellular lipids of microalgae were hardly extracted from wet biomass through solvents without cell disintegration. Ultrasound causes apoptosis in microalgae cells and bubbles in solvent, acting to release lipid/oil [6]. Chloroform/methanol mixture contains both polar (methanol) and non-polar (chloroform) solvents that can extract either neutral (generally storage) or polar (generally membrane-associated) lipids [53]. Adam and Abert-Vian (2012) showed that the application of ultrasound was favorable for extraction at an industrial scale [18].

CONCLUSIONS

This study highlighted the potential application of high frequency (1.7 MHz) ultrasound wave in biomaterial extraction from green algae and discussed its effects on different irradiation methods with exposure time. Direct irradiation of ultrasound caused a significant increase in chlorophylls extraction yield, while there was no remarkable difference between the results of the indirect exposure to the ultrasound waves and those of the control system. The yield of carotenoid reached 66% and 119% in the direct and indirect exposure to ultrasonic irradiations, respectively. The results showed that ultrasound waves can degrade the released bio-substances such as carotenoid. The bio-substances extraction enhancement was due to the production of cavitation bubble through ultrasound wave propagation. Although using ultrasound can increase the extraction and isolation of intracellular metabolites yields, the structure of biodrugs plays an important role in pharmaceuticals. Therefore, the method of irradiation and other factors affecting the irradiation methods of ultrasound wave should be considered in ultrasound technique to obtain the maximum yield in biodrug isolation from microalgae.

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