Optimization of cell wall disruption and lipid extraction methods by combining different solvents from wet microalgae

Running title: Extraction of lipids from wet microalgae

Daryush Arabian[1]
1-University Institute of Applied Science, Malek Ashtar University of Technology, Isfahan, Iran.
darabian@mut.ac.ir

Abstract:

Microalgae have emerged as one of the most promising options for biodiesel production over the past few decades. Lipid extraction from microalgae for biodiesel production as a bottleneck of biodiesel production technology was the main purpose of this study. In this study different methods of the cell wall disruption were compared. Then, two methods of ultrasound and bead mill were used as methods of the cell wall disruption. The maximum lipid extracted by ultrasound was 17.10% and by bead mill was 15.16% (based on microalgae biomass dry weight). After the cell wall disruption of microalgae, for lipid extraction, chloroform-methanol solvent combination was used as a high extraction method and hexane-ethanol solvent combination was used as an environmentally friendly method. In this regard, the effect of solvent to biomass ratio, temperature and extraction time was investigated and the optimal results for chloroform-methanol solvent combination were 8 ml/g, 45°C and 60 minutes, respectively, and for hexane-ethanol combination were 6 ml/g, 35◦C and 73 minutes, respectively. Under these optimal conditions, the highest amount of extracted lipid from *Chlorella vulgaris* with a moisture content of 87.50%, and ultrasound as a cell wall disruption method were obtained 20.39% and 16.41% (based on microalgae dry weight) with a combination of chloroform-methanol solvents and hexane-ethanol respectively. Also the highest extraction rates of 17.63% and 13.85% were obtained for the combination of chloroform-methanol and hexane-ethanol solvents, respectively by bead milling as cell wall disruption method.

Keywords: Bead mill, *Chlorella vulgaris*, Lipid, Microalgae, Solvent extraction, Ultrasound.

1- Introduction

Lipid extraction methods can be divided into two categories: wet and dry extraction, based on the method of microalgae biomass preparation (Ghasemi Naghdi et al. 2016; Yang et al. 2014). Also based on the method of extraction, can be divided into two categories; mechanical and chemical or a combination of these two (Ranjith Kumar, Hanumantha Rao, and Arumugam 2015). In wet extraction methods, microalgal cell disruption occurs after initial condensation and in conditions where the biomass still contains a large amount of residual water (Ghasemi Naghdi et al. 2016; Yang et al. 2014). While in dry methods, mechanical or chemical destruction of cells is done after dehydration of microalgae and bringing the biomass moisture to about 10%, (Browne et al. 2009; Singh and Gu 2010). From mechanical extraction methods, extraction by ultrasound and osmotic shock and among the chemical methods extraction with solvent can be mentioned (McMillan et al. 2013). The properties of microalgae cell wall play an important role in choosing the appropriate method for lipid extraction due to its high resistance (Prabakaran and Ravindran 2011). The effect of different methods on different species of microalgae will not be the same, depending on the size, shape and structure of their cell wall. Also, lipid extraction from microalgae is greatly affected by the small size of microalgae cells and the amount of moisture in them. The choice of the appropriate method for lipid extraction adepends on the physical state of

the biomass, or more precisely the wet or dry state of the biomass (Taher et al. 2014).Therefore, determining the most efficient and at the same time cost-effective method, which is also acceptable in terms of environmental impact, is a major challenge to the lipid extraction process (Halim, Danquah, and Webley 2012). Lipid extraction from microalgae should be fast and effective to preventing the destruction of lipids or fatty acids (Medina et al. 1998). Ideally, in order to minimize the extraction of non-lipid contaminants such as lipid-associated proteins and carbohydrates, the technology used in extraction should be able to selectively extract lipids (Prabakaran and Ravindran 2011). Also, in order to reduce the separation and purification operations in downstream, the technology should be more inclined to extract glycerides than other types of lipids such as polar and non-polar lipids that cannot be easily converted to biodiesel. In addition, the selected technology must be efficient (both in terms of energy and time), unresponsive to lipids, relatively cheap and safe (Halim, Danquah, and Webley 2012). In lipid extraction from microalgae, the amount of moisture in microalgae is one of the most important parameters (Tanzi, Vian, and Chemat 2013). According to one hypothesis, the presence of residual water in the biomass improves the efficiency of lipid extraction. Because water swells the cells and allows the solvent to access the lipids. According to this hypothesis, drying of concentrated microalgae before lipid extraction is unnecessary and may prevent lipid mass transfer (Halim, Danquah, and Webley 2012). Dehydration and drying of microalgae biomass, on a large scale, requires a large surface area, spend more time and energy, therefore selecting and using technology that can extract lipids directly from wet biomass is a great economic advantage (Sander and Murthy 2010; Halim, Danquah, and Webley 2012). Hence, processes based on wet biomass extraction are superior to dry biomass extraction processes due to the lower energy consumption for drying. Also, in the drying process, in addition to high cost, the high temperature used during the drying process may affect the composition as well as the lipids obtained from algal biomass and cause the loss of lipids due to degradation (Iqbal 2012).

There are several methods for extracting lipids from microalgae, including solvent extraction and supercritical extraction (Taher et al. 2014). Also, pre-preparation methods are usually used in conjunction with solvent extraction to break down the cell wall. In fact, the pre-preparation stage is before extraction, which reduces the operation time and often increases the amount of obtained product. In this study, the methods of the cell wall disruption as well as extraction of lipid by solvent from wet microalgae was optimized using two different solvent combinations.

2- Material and methods

2-1- Pre-culture and micro-algae culture medium of *C. vulgaris*

Inoculation was carried out by the native species of *C. vulgaris* microalgae equipped from the National Center for Aquatic Processing Research, Bandar Anzali, Iran, at a rate of 10% in the Zarrouk culture medium consists of (part A) $NaHCO_3$ 16.80 g and K_2HPO_4 0.50 g; (part B) $NaNO_3$ 0.50 g, K_2SO_4 1.00 g, NaCl 1.00 g, $MgSO_4 \cdot 7H_2O$ 0.20 g, EDTA-$Na_2 \cdot 2H_2O$ 0.08 g, $CaCl_2 \cdot 2H_2O$ 0.04 g, and $FeSO_4 \cdot 2H_2O$ 0.01 g; trace elements mixture A (part C 10 mL/l): 1.00 mL, trace elements mixture B (part D 1.0 mL/l): 1.00 mL; part C mg/L: H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.810 g, $ZnSO_4 \cdot 7H_2O$ 0.222 $MoO_3 \cdot 0.015$, and $CuSO_4 \cdot 5H_2O$ 0.074 (the used amount is 10 mL/l); part D mg/L: NH_4VO_3 22.9, $NiSO_4 \cdot 7H_2O$ 47.8, $NaWO_2$ 17.9, $Ti_2(SO_4)_3 \cdot 6H_2O$, and $Co(NO_3)_2 \cdot 6H_2O$ 4.4 (the amount used was 1.0 mL/l) (Medina et al. 1995). The culture was incubated at 130 rpm, with a temperature of 30 °C and a light intensity of 2500 lux. They were kept up to optimal growth. As a control sample a culture medium without microalgae inoculation was used. Photobioreactors were made with the help of 5-liter plastic gallons. For aeration, air compressor equipped with CO_2 cylinder were used. To better distribute the air inside the photobioreactor a spargers was used, which was connected to the air compressor via a silicone hose (Fig. 1). An aeration rate of 0.5 vvm with 3 % CO_2 was continuously provided for all treatments. For the main culture, about three liters of the culture medium with the 7 to 7.5 pH were poured into each photobioreactor and 300 ml of microalgae solution (10 % inoculation) was added to the photobioreactors. A control sample without microalgae inoculation was also used. The photobioreactors were exposed to sunlight, and the growth rate of the microalgae was measured regularly.

2-2- Measure the growth rate of microalgae and draw a growth curve

Turbidity measurement method was used to measure microalga growth. In this method, 10 ml of culture medium was removed daily and its turbidity was measured by spectrophotometer at a wavelength of 600 nm. Then, according to the amount of absorbance, and according to the obtained values, its growth curve was drawn.

2-3- Measuring the moisture content of concentrated microalgae

Dry weight method was used to measure the moisture content of concentrated miroalgae. 1 g of concentrated microalgae was weighed (W_1) and poured into a pre-weighed empty micro tube (W_2). The microtubule was then placed in an oven at 60°C for 24 hours, after the sample was completely dried, the micro tube was weighed (W_3). From the difference in weight of the empty micro tube and the micro tube containing the dried microalgae (W_3-W_2), the weight of the dried microalgae (W_4) is obtained. The following equation was used to obtain the percentage of water in the concentrated microalgae. The moisture content of microalgae was about 87.5%.

$$Moisture\% = \left[\frac{(W1 - W4)}{W1}\right] \times 100 \qquad \text{(Eq.1)}$$

2-4- Investigation of different methods of the cell wall disruption by measuring the total chlorophyll content

5 methods of ultrasonic waves with 28 KHz and 300 W (watts) for 30 min, osmotic shock with 10% NaCl for 48 hr, microwave waves with 2450 MHz and 1000 W for 10 min, high speed bead mill (400 rpm for 30 min) and liquid nitrogen were used as cell wall disruption methods. For

each method, 5 g of the sample was weighed and then 20 ml of 90% methanol was added to each sample. After performing cell disruption procedures under the mentioned conditions, the samples were kept in the dark for 24 hr at 4 ° C. To separate the microalgae biomass from the solvent, the samples were centrifuged at 5,500 rpm (1507 g) for 5 min. 1 ml was taken from each sample and diluted with 90% methanol up to 10 times. The absorption rate of the samples is obtained with a spectrophotometer in the wavelength range of 300 to 700 nm. According to the data obtained from the equations, the total chlorophyll content for each sample is calculated.

Chlorophyll a: 16.5 A665 – 8.3 A650 Eq. 2

Chlorophyll b: 33.8 A650 – 12.5 A665 Eq. 3

Total chlorophyll (a+b): 4 A665 + 25.5 A650 Eq. 4

2-5- Investigation of different methods of microalgae cell wall breakdown by measuring the amount of extracted lipid

After performing cell wall disruption methods, the amount of extracted lipid for each method was measured by the standard Bligh and Dyer protocol. A mixture of chloroform and methanol (1:1 v/v) was added to cells for lipid extraction. The lower layer containing the extracted lipid and chloroform solvent was separated. The solvent was removed by evaporation and lipid content was measured gravimetrically (Bligh and Dyer 1959). The lipid productivity (P_{lipid}) was determined based on the calculation indicated in (Eq. 5) (Yeh and Chang 2012):

$$P_{lipid}\left(\frac{mg}{l.d}\right)=\frac{\text{cumulative microalgae biomass production }(mg)\times\text{lipid content (\%)}}{\text{working volume }(l)\times\text{cultivation time (d)}}$$

(Eq. 5)

2-6- Investigating the effect of the time of using ultrasound waves

An ultrasonic bath device with a frequency of 28 kHz and a power of 300 watts was used at different times. Thus, for one gram of microalgae, 5 ml of water was used as a solvent. The sample containing water and microalgae was poured into 50 ml tubes and the tubes were placed in the chamber of the device. In the time interval between 5 to 30 minutes with the design of the experiment performed with the single factor$_1$ section of Design Expert 10.0.0 software, which is specified in Table 2, ultrasound waves were used. After cell wall disruption, the samples were centrifuged at 3000 (1107 g) rpm for 5 minutes. Then, according to the standard Bligh and Dyer protocol, lipid extraction was performed.

2-7- Optimization of microalgal cell wall disruption using bead mill

A Fritsch bead mill with double 250 ml steel cups by steel beads with a fixed diameter of 5 mm was used. Four parameters of shaft rotation speed (cup rotation speed), milling time, cell suspension concentration and pellet volume to cup volume ratio were selected to evaluate their effect on lipid extraction rate. The range of each of the mentioned parameters according to the sources and limitations of using the device were selected (Table 1). According to the design of the experiment performed with the CCD_2 section of Design Expert 10.0.0 software, which is shown in Table 1, the tests were performed for each of the specified conditions. After milling, the samples were centrifuged at 3000 g (1107 g) for 5 minutes. Then, according to the standard protocol of Bligh and Dyer, lipid extraction was performed with solvent to achieve optimal conditions.

2-8- Optimization of lipids extraction from microalgae

The method discussed in this study for lipid extraction from microalgae is solvent extraction method. The combination of chloroform-methanol solvents and the hexane-ethanol solvents were used for comparison. After performing cell wall disruption methods and reaching optimal conditions, a mixture of both solvent pairs was used for each cell wall disruption method. Among the parameters affecting solvent extraction, extraction time and temperature and solvent to biomass ratio were selected to investigate and reach the optimal conditions and the parameters of non-polar solvent to aqueous solution and water content in aqueous solution were considered 0.5 and 40% for the combination of chloroform-methanol-water solvents, respectively, and 0.7 and 35% for the combination of hexane-ethanol-water solvents, respectively.

2-8-1- The effect of solvent to biomass ratio on the rate of lipid extraction

In order to minimize the amount of solvent consumption and to achieve the optimal ratio of solvent to biomass, its effect on the rate of lipid extraction was investigated. Therefore, one gram of wet biomass and 2, 4, 6, 8 and 9 ml of solvent were used.

2-8-2- Investigation of the effect of temperature and time on lipid extraction

To investigate the effect of temperature and time on the amount of lipids extraction, these parameters were considered in the range listed in Table 2. According to the design of the experiment performed with the CCD part of Design Expert 10.0.0 software, which is shown in Table 2, the experiments were performed for each of the specified conditions.

3- Results

3-1- Investigation of different methods of microalgal cell wall disruption

As mentioned before, cell wall disruption as a preparative method has a great effect on increasing the amount of lipids extraction. Accordingly 5 methods of ultrasound, osmotic shock, microwave waves, bead mill and liquid nitrogen were used as cell wall disruption methods and also a sample without cell wall disruption was used as a control. The two methods of measuring total chlorophyll content and measuring the amount of extracted lipid were used as a criterion for comparing the rate of cell disruption in different methods.

3-1-1- Investigation of different methods of microalgae cell wall disruption by measuring the total chlorophyll content

The adsorption results obtained from the samples at a wavelength of 300 to 700nm were plotted in Fig. 2 and the total chlorophyll content was calculated from the equations 2-4, the results of which are shown in Fig. 3.

Comparing the obtained results with the control sample with the total chlorophyll content of 1.75 mg/l, the highest total chlorophyll content were obtained respectively, by ultrasound with 7.5 mg/l, bead mill with 6.16 mg/l, osmotic shock 4.58 mg/l, microwave waves 4.22 mg/l and liquid nitrogen with 2.90 mg/l.

3-1-2 Evaluation of different methods of microalgal cell wall disruption by measuring the amount of extracted lipid

After performing the cell wall disruption methods mentioned in the previous section, the amount of extracted lipid for each method was measured by the standard Bligh and Dyer protocol. The results are shown in Fig. 4. By comparing the obtained results to the control sample with the amount of 5.47% extracted lipid (percentage of dry weight) the highest amount of extracted lipids was obtained respectively by ultrasonic methods with 16.93%, microwave with 15.07%, bead mill with 14.31%, osmotic shock with 11.25% and liquid nitrogen with 2.8%. Comparing the results obtained from the both measuring methods, except for the differences in the use of microwaves, the order of the effect of cell wall disruption methods is the same in both studies. These results are consistent with results of other studies (Prabakaran and Ravindran

2011; Wang et al. 2014; Montalescot et al. 2015; Gerde et al. 2012). It seems that for more accurate calculation, the method of measuring the amount of total chlorophyll can be used as a criterion for comparing the microalgae cell wall disruption methods along with measuring the amount of extracted lipid.

3-2- Optimization of microalgae cell wall disruption using ultrasound

In this study, time was considered as the only parameter for optimization, the single factor part of Design Expert 10.0.0 was used and the time was considered between 5 to 30 minutes. The results are given in Table 3. According to the data in Table 3, the highest rate of lipid extraction is related to experiment 3 with a value of 10.17%. The data has been analyzed by the software and the outputs are shown in Fig. 5. This model, as reported in its report, with F-value = 111.86, R^2 = 0.9781 and p-value less than 0.0001 is quite stable and significant. Given the value of 0.9694 for Adj R-Squared, which is the result of experiments, the value predicted by the model, ie 0.831, is a suitable value for Pred R-Asquared, which indicates the accuracy and reliability of the designed model based on the test data. Adeq Precision indicates the signal-to-noise ratio, which is a ratio of more than 4, and in this experiment the ratio of 22.982 indicates a very good ratio, and as a result this model can be used to guide the proper design.

As shown in and Fig. 5, lipid extraction efficiency increases by increasing the time of ultrasound but this increase is up to 30 minutes, after which the extraction efficiency does not increase, and excess time only increases energy consumption and thus increases costs. Also, increasing the time increases the temperature and as a result, it changes the structure of the lipid and reduces the rate of lipid extraction. Therefore, the optimal conditions for using ultrasound at 28 kHz and 300 watts, with a solvent to biomass ratio of 5:1, is 30 minutes.

According to the contents and the analysis performed by the software based on the laboratory information obtained for the amount of lipid extraction, the model proposed by the software is a quadratic model. The equation is generally as follows:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_{21} + \beta_{22}X_{22} + \beta_{33}X_{23} \quad \text{(Eq. 6)}$$

In this regard, Y is the answer that is predicted. B_0 is a constant value X_1, $X_2$and X_3 are independent values and β_1, β_2 and β_3 are coefficients that determine the linear values of X_1, X_2 and X_3, respectively. The coefficients β_{12}, β_{13} and β_{23} express the interactions between the variables and the coefficients β_{11}, β_{22} and β_{33} are coefficients that express the quadratic effects of the variables X_1, X_2 and X_3. The proposed equation for this model is as follows:

$$\text{Lipid extraction} = 5.60127 + 0.54523A - 6.25829e\text{-}003A^2 \quad \text{(Eq. 7)}$$

3-3- Optimization cell wall disruption using bead mill

Among the effective parameters, four parameters of cup rotation speed, milling time, cell suspension concentration and bead volume to cup volume ratio were considered. The results are given in Table 4.

The model considered for the mentioned responses in the table 4 has been identified as a quadratic model by examining R^2 and the reliability of all possible cases. The overall capability of the model is typically described by measuring the R^2 coefficient and is a measure of the model's versatility. But the coefficient R^2 alone is not enough to confirm the model hence the analysis of variance for the model is performed. The details are given in Table 5. This model, as reported in its report, with F-value= 208.96 and R^2= 0.9893 (indicates the conformity of the selected model is 98.93%) and p-value less than 0.0001 is completely stable and meaningful. In

this model, the parameters A, B, C, D, AB, A^2, B^2 and D^2 with a p-value less than 0.05 indicate the prevalence of that factor and are important. The relative importance of all parameters as well as the interaction between them in the final model is presented by the impact factor of each in the model.

The value of 09846 for Adj R-Squared, which is the result of experiments, the value predicted by the model, 0.9563, is a suitable value for Pred R-Asquared, which indicates the correctness of the designed model. As can be seen from the F-value results in analysis of variance, velocity has the greatest effect on extraction efficiency, followed by the ratio of bead volume to cup volume, milling time and cell suspension concentration, respectively. According to the one-dimensional surface diagrams, the individual parameters are discussed.

According to Fig. 6, as the velocity increases, the efficiency of lipid extraction increases with a steep slope. Higher velocities, increase the number of collisions per unit time, resulting in more energy being transferred to the biomass particles and causing more microalgal cell wall disruption. According to the other studies, the use of higher speeds increases the amount of extracted lipids, reduces milling time and thus reduces costs (Lee et al. 2010). It should also be noted that increasing the velocity to a critical speed is possible. At this speed, the pellets adhere to the inner wall and do not hit the particles inside the cup, so the maximum velocity must be less than this critical speed. On the other hand, at high speeds, the temperature of the container and pellets rises, which can change the structure of the lipid and thus reduce the rate of lipid extraction. As shown in Fig. 7, with increasing milling time to about 30 minutes, the lipid extraction efficiency increases with a slow slope and does not increase after that. In other words, higher times do not have the effectiveness to improve the extraction efficiency (Montalescot et al. 2015). As mentioned, increasing time, especially at high speeds, causes an increase in temperature, a change in lipid structure, and thus a decrease in efficiency, as well as an increase in energy consumption (Lee et al. 2010). From Fig. 8, it can be seen that with increasing the concentration of cell suspension, the extraction efficiency does not increase significantly or in other words, this parameter has little effect on increasing the amount of extracted lipid. In comparison in study by Postama et al., increasing biomass concentration, was found to have positive effects on *Chlorella vulgaris* cell disruption efficiency (Postma et al. 2015), In contrast, the negative impact of this increase has also been reported (Doucha and Lívanský 2008). By examining the effect of the ratio of pellet volume to cup volume in the results and the diagram of Fig. 9, it can be seen that increasing this ratio to about 60% (v/v), increases the lipid extraction efficiency and in higher ratios due to the lack of enough space for the pellets to rotate, the number of collisions per unit time decreases and as a result less energy enters the sample, which ultimately does not increase the efficiency of lipid extraction. Increasing this ratio has been reported to increase cell disruption kinetics (Moreno-Garrido 2008). Also, some authors have introduced relationships between cell disruption and bead collision frequencies (Moreno-Garrido 2008; Melendres et al. 1991) and have found correlations between bead collision frequency and product release rate, sometimes leading to the parameters optimization.

According to the results of analysis of variance, only the speed and time parameters have significant interactions which are shown as surface diagrams. In fact, surface diagrams are three-dimensional diagrams that are drawn as a function of two different independent variables in the range of experiments, while the other variables are on a fixed surface. According to the curve in Fig. 10, the extraction efficiency increases with increasing rotation speed at shorter milling times. Thus, as can be seen in the curve of Fig. 10, both mentioned parameters are in their optimal range in the intervals of 500> rotation speed> 400 and 25> time> 17.5. In particular, the

maximum efficiency is 15.6%. For a speed of 500 rpm, a time of 20 minutes, with a ratio of cell suspension concentration of 20% and a ratio of bead volume to cup volume of 40% was obtained.

According to the analysis performed by the software based on the laboratory information obtained, the amount of lipid extraction based on dry weight can be calculated by the following equation:

$$\text{Lipid extraction} = 4.43598 + 0.0471866A + 0.21436B + 0.013110C + 0.13056D - 2.26625e\text{-}004AB - 3.40268e\text{-}005A^2 - 2.29207e\text{-}003B^2 - 3.67560e\text{-}004C^2 - 1.19970e\text{-}003D^2 \quad \text{(Eq. 8)}$$

3-4- Optimization of lipids extraction by solvent from microalgae - pretreated by ultrasound and bead mill

The optimization results showed that the behavior of lipid extraction with solvent is the same in both pretreatment methods (bead mill and ultrasound) and the optimal points obtained for both methods are the same. Using the optimal conditions obtained for ultrasound waves, the bead mill, microalgal cell wall disruption was performed. Afterwards, lipid extraction from microalgae was performed by combining chloroform-methanol-water and hexane-ethanol-water solvents with the aim of optimizing the three parameters of biomass to solvent ratio, extraction time and temperature. According to the results of Table 6, the highest amount of extracted lipid in ultrasound pretreated testes were 20.39% and 16.41% with hexane-ethanol and chloroform-methanol solvents respectively. The same for bead mill testes were 13.85% and 17.63%.

Table 7 shows the analysis of variance of the data related to lipid extraction with chloroform-methanol and hexane-ethanol solvents. In this models, parameters A, B, C and C^2 are important with p-value less than 0.05. According to the results, the solvent to biomass ratio parameter has the highest impact on lipid extraction efficiency and the parameters; extraction time and temperature are significant afterwards. Yang et al., also investigated the optimum conditions of lipid extraction and their results revealed that the solvent to biomass ratio had the largest effect on lipid extraction efficiency, followed by extraction time and temperature (Yang et al. 2014).

According to Fig. 11, it is clear that with increasing extraction time, lipid extraction efficiency also increases, but this increase occurs with a slow slope, for example, with increasing extraction time from 20 to 80 minutes, lipid extraction efficiency increases by approximately 3%. At higher times, the desired mass transfer between the solvent and the biomass does not occur. By examining the increase in time from 1 to 24 hours, it has been found that the extraction efficiency increases by about 1 to 2 percent. Increasing the time will only increases energy consumption and cost. According to the results, the optimal time for extraction are 60 and 70 minutes for chloroform-methanol and hexane-ethanol solvents respectively. In Yang et al., study also further increase in time of extraction had no effect on the extraction and 37 min time was reported as the optimal value of extraction (Yang et al. 2014).

Fig. 12 shows the effect of temperature on the amount of extraction in, it can be seen that increasing the temperature does not significantly increase the amount of lipid extraction and also the use of higher temperatures causes changes in cell content and lipid structure (Ranjith Kumar, Hanumantha Rao, and Arumugam 2015). It may also cause part of the solvent to evaporate, which reduces lipid extraction. In most laboratory operations, lipid extraction with solvent is usually performed at room temperature. According to the results, the optimum temperature was 45◦c and 35◦c for extraction with chloroform-methanol and hexane-ethanol solvents respectively

The effect of biomass to solvent ratio parameter on lipid extraction is shown in Fig. 13. As mentioned before, this parameter has the highest impact on the extarction. According to the figure, this increase is accompanied by a steep slope, for example, by increasing this ratio from 2 to (ml/g) 8, the amount of extracted lipid increases by 13%. However, this increase is up to the ratio of 8 (ml/g) and further increase of this ratio has no effect on increasing the efficiency of lipid extraction. Yang et al., also established that excess solvent amount would not improve further the extraction yield (Yang et al. 2014). Therefore, the optimal ratio of 8(ml/g) and 6(ml/g) for extraction with chloroform-methanol and hexane-ethanol solvents were obtained respectively.

According to the results of analysis of variance, only the parameters of extraction time and the ratio of solvent to biomass have a relatively significant interaction which is shown in Fig. 14. According to the curve of Fig. 14, with increasing the ratio of biomass to solvent and increasing the extraction time, the extraction efficiency increases. According to the results, in 60 minutes, temperature 35 ◦c and solvent to biomass ratio 8 (ml/g), the maximum yield was 20.39%. The both mentioned parameters are in their optimal range in the intervals of 8 > solvent to biomass ratio> 5 and 70> time> 45. According to the contents and analysis performed by the software based on laboratory information obtained, the amount of lipid extraction can be calculated by the following equation.

Lipid extraction by chloroform-methanol solvents = 3.13229 - 1.80469e-003A - 0.026270B + 2.82282C +4.50000e-003 AC - 0.13723 C^2 (Eq. 9)

Lipid extraction by hexane-ethanol solvents = 9.2091 + 0.074717A + 0.035034B + 6.21279C - 7.12500e-003 AC – 0.42124C^2 (Eq. 10)

4- Conclusion:

In this research, the extraction methods was optimized with ultrasound and bead mill as cell disruption methods and chloroform-methanol and hexane-ethanol as different solvent extraction process. The optimal time for using ultrasound as a microalgae cell wall disruption method was 30 minutes at 28 kHz, 300 watts and the solvent to biomass ratio was 5:1. The highest amount of extracted lipid in this condition was 17.10%. Optimal conditions for bead milling were obtained, speed 400 rpm, time 30 minutes, cell suspension concentration 30% and bullet volume to cup volume ratio 60%. The highest amount of extracted lipid under these conditions, was obtained 14.57%. Despite the good performance of ultrasound in destroying the cell wall of most microalgae, it seems that due to high operating costs, it is not possible to use this process on an industrial scale at present. It seems that among the methods of preparation and destruction of cell walls, the bead mill method has more potential for use on industrial scales. Therefore, it is better to use a bead mill with higher speeds and in a shorter time to increase efficiency and reduce energy costs.

In solvent extraction method, three factors such as temperature and time of extraction and solvent to biomass ratio were optimized, solvent to biomass ratio had the highest impact and the extraction temperature, had the least effect on the extraction of lipids by both chloroform-ethanol and hexane-ethanol combined solvents. The use of chloroform-methanol combination, although it has a higher lipid extraction efficiency and is a suitable method for lipid extraction on a laboratory scale, but due to the toxicity and high price of chloroform, it cannot be a suitable option for lipid extraction on an industrial scale. Although the hexane-ethanol combination is less efficient than the methanol chloroform, it is less toxic and more expensive than the chloroform-methanol combination and is more environmentally friendly on an industrial scale. Also, due to the fact that the contents inside the microalgae cell, especially lipids, are sensitive to

temperatures above 70 ° C, and also at higher temperatures, depending on the type of solvents used, there is a possibility of solvent evaporation and thus reducing lipid extraction efficiency. To reduce energy consumption, it is better to extract lipids at room temperature.

References:

Bligh, E Graham, and W Justin Dyer. (1959). 'A rapid method of total lipid extraction and purification', *Canadian journal of biochemistry and physiology*, 37: 911-17.

Browne, Ben, Ryan Gibbs, Jackson McLeod, Michael Parker, Wade Schwanda, and Kyle Warren. (2009). 'Oil extraction from microalgae', *Algae oil extraction capstone*, 2010.

Doucha, J, and K Lívanský. (2008). 'Influence of processing parameters on disintegration of Chlorella cells in various types of homogenizers', *Applied microbiology and biotechnology*, 81: 431-40.

Gerde, Jose A, Melissa Montalbo-Lomboy, Linxing Yao, David Grewell, and Tong Wang. (2012). 'Evaluation of microalgae cell disruption by ultrasonic treatment', *Bioresource technology*, 125: 175-81.

Ghasemi Naghdi, Forough, Lina M González González, William Chan, and Peer M Schenk. (2016). 'Progress on lipid extraction from wet algal biomass for biodiesel production', *Microbial biotechnology*, 9: 718-26.

Halim, Ronald, Michael K Danquah, and Paul A Webley. (2012). 'Extraction of oil from microalgae for biodiesel production: A review', *Biotechnology advances*, 30: 709-32.

Iqbal, Javed. (2012). 'Development of cost-effective and benign lipid extraction system for microalgae'.

Lee, Jae-Yon, Chan Yoo, So-Young Jun, Chi-Yong Ahn, and Hee-Mock Oh. (2010). 'Comparison of several methods for effective lipid extraction from microalgae', *Bioresource technology*, 101: S75-S77.

McMillan, Jonathan R, Ian A Watson, Mehmood Ali, and Weaam Jaafar. (2013). 'Evaluation and comparison of algal cell disruption methods: microwave, waterbath, blender, ultrasonic and laser treatment', *Applied energy*, 103: 128-34.

Medina, A Robles, A Giménez Giménez, F García Camacho, JA Sánchez Pérez, E Molina Grima, and A Contreras Gómez. (1995). 'Concentration and purification of stearidonic, eicosapentaenoic, and docosahexaenoic acids from cod liver oil and the marine microalgaIsochrysis galbana', *Journal of the American Oil Chemists' Society*, 72: 575-83.

Medina, A Robles, E Molina Grima, A Giménez Giménez, and MJ Ibáñez González. (1998). 'Downstream processing of algal polyunsaturated fatty acids', *Biotechnology advances*, 16: 517-80.

Melendres, Ariel V, Hiroyuki Honda, Naohiro Shiragami, and Hajime Unno. (1991). 'A kinetic analysis of cell disruption by bead mill', *Bioseparation*, 2: 231-36.

Montalescot, V, T Rinaldi, R Touchard, S Jubeau, M Frappart, P Jaouen, P Bourseau, and L Marchal. (2015). 'Optimization of bead milling parameters for the cell disruption of microalgae: process modeling and application to Porphyridium cruentum and Nannochloropsis oculata', *Bioresource technology*, 196: 339-46.

Moreno-Garrido, Ignacio. (2008). 'Microalgae immobilization: current techniques and uses', *Bioresource technology*, 99: 3949-64.

Postma, PR, TL Miron, G Olivieri, MJ Barbosa, RH Wijffels, and MHM Eppink. (2015). 'Mild disintegration of the green microalgae Chlorella vulgaris using bead milling', *Bioresource technology*, 184: 297-304.

Prabakaran, Pandian, and A David Ravindran. (2011). 'A comparative study on effective cell disruption methods for lipid extraction from microalgae', *Letters in applied microbiology*, 53: 150-54.

Ranjith Kumar, Ramanathan, Polur Hanumantha Rao, and Muthu Arumugam. (2015). 'Lipid extraction methods from microalgae: a comprehensive review', *Frontiers in Energy Research*, 2: 61.

Sander, Kyle, and Ganti S Murthy. (2010). 'Life cycle analysis of algae biodiesel', *The International Journal of Life Cycle Assessment*, 15: 704-14.

438 Singh, Jasvinder, and Sai Gu. (2010). 'Commercialization potential of microalgae for biofuels production',
439 *Renewable and sustainable energy reviews*, 14: 2596-610.
440 Taher, Hanifa, Sulaiman Al-Zuhair, Ali H Al-Marzouqi, Yousef Haik, and Mohammed Farid. (2014).
441 'Effective extraction of microalgae lipids from wet biomass for biodiesel production', *Biomass*
442 *and bioenergy*, 66: 159-67.
443 Tanzi, Celine Dejoye, Maryline Abert Vian, and Farid Chemat. (2013). 'New procedure for extraction of
444 algal lipids from wet biomass: A green clean and scalable process', *Bioresource technology*, 134:
445 271-75.
446 Wang, Meng, Wenqiao Yuan, Xiaoning Jiang, Yun Jing, and Zhuochen Wang. 2014. 'Disruption of
447 microalgal cells using high-frequency focused ultrasound', *Bioresource technology*, 153: 315-21.
448 Yang, Fangfang, Wenzhou Xiang, Xiumei Sun, Hualian Wu, Tao Li, and Lijuan Long. (2014). 'A novel
449 lipid extraction method from wet microalga Picochlorum sp. at room temperature', *Marine drugs*,
450 12: 1258-70.
451 Yeh, Kuei-Ling, and Jo-Shu Chang. (2012). 'Effects of cultivation conditions and media composition on
452 cell growth and lipid productivity of indigenous microalga Chlorella vulgaris ESP-31',
453 *Bioresource Technology*, 105: 120-27.

454

455

456

457 Table 1- Parameters and levels for optimization of microalgal cell wall disruption using bead
458 mill

Parameters	Minimum (-1)	Maximum (+1)
rotation speed (rpm)	200	400
milling time (min)	10	30
cell suspension concentration (%wt)	10	30
pellet volume to cup volume (%v/v)	20	60

459 Table 2- Parameters and levels for optimization of lipids extraction from *Chlorella vulgaris*

Parameters	Minimum (-1)	Maximum (+1)
Extraction temperature (^oC)	25	45
Time of extraction (min)	20	60
solvent to biomass ratio (ml/g)	4	8

460

461 Table 3. Lipid extraction results from experimental design to optimize the use time of ultrasound

Test No.	Time of using ultrasound (5-30 min)	Extracted lipid (%dry wt)
1	17.5	12.43
2	5	8.67
3	30	17.10
4	24.15	14.72
5	5	8.02
6	11.25	10.85
7	30	16.88

8	40	16.91

462 Table 4- Lipid extraction results from experimental design for bead mill optimization

Test	rotation speed (rpm)	milling time (min)	cell suspension concentration (%wt)	pellet volume to cup volume(%v/v)	Extracted lipid (%dry wt)
1	300	20	20	40	12.55
2	500	20	20	40	15.16
3	200	10	30	20	7.82
4	200	30	10	60	10.5
5	300	20	20	80	12.27
6	400	30	10	60	14.31
7	400	10	30	60	13.89
8	400	10	10	20	12.17
9	200	30	30	20	8.84
10	300	20	20	40	12.18
11	400	30	30	20	13.11
12	200	30	10	20	8.74
13	300	20	20	40	12.26
14	400	30	30	60	14.57
15	300	20	20	40	12.32
16	300	20	20	40	12.41
17	100	20	20	40	6.95
18	300	40	20	40	12.75
19	400	10	10	60	13.56
20	300	20	40	40	12.72
21	200	10	30	60	9.41
22	300	20	20	40	12.47
23	200	30	30	60	10.72
24	200	10	10	60	8.89
25	400	10	30	20	12.36
26	400	30	10	20	12.97

463 Table 5- Analysis of variance related to bead mill optimization

	df	Sum of squares	Mean square	F Value	p-value	
Model	8	132.14	16.52	208.96	< 0.0001	Significant
rotation speed (A)	1	108.23	108.39	1369.33	< 0. 0001	-
milling time (B)	1	4.45	4.45	56.12	< 0.0001	-
cell suspension concentration (C)	1	0.38	0.38	4.74	0.0429	-
pellet volume to cup volume (D)	1	11.29	11.29	141.03	< 0.0001	-

AB	1	0.84	0.84	10.42	0.0047	-
A^2	1	3.50	3.50	44.30	< 0.0001	-
B^2	1	0.81	0.81	10.25	0.0049	-
D^2	1	3.35	3.35	41.95	< 0.0001	-
Residuals	18	1.42	0.079	-	-	-
Lack of fit	13	1.34	0.13	11.23	0.0625	insignificant
Pure error	5	0.45	0.076	-	-	
Cor Total	26	133.52	-	-		-

Std. Dev.	0.29	R-Squared	0.9893
Mean	11.63	Adj R-Squared	0.9846
C.V. %	2.46	Pred R-Squared	0.9563
PRESS	5.83	Adeq Precision	52.333

464

465 Table 6- Results of optimization of lipid extraction by solvents

test	Time of extraction 20-60 (min)	Temperature 25-45 (c)	Solvent to biomass ratio (ml/g)	Lipid extraction efficiency by Hexane-ethanol solvent (%dry wt) Pretreated by ultrasound	Lipid extraction efficiency by chloroform-methanol solvent (%dry wt) Pretreated by ultrasound	Lipid extraction efficiency by hexane-ethanol solvent (%dry wt) Pretreated by bead mill	Lipid extraction efficiency by chloroform-methanol solvent (%dry wt) Pretreated by bead mill
1	6.36	35	6	14.12	15.80	11.90	13.51
2	40	35	6	15.7	17.23	12.97	14.7
3	40	35	6	15.84	16.72	13.44	14.86
4	74	35	6	16.41	17.92	13.85	5.5
5	40	35	6	15.61	16.97	12.83	15.16
6	40	18	6	15.16	16.32	12.59	14.63
7	60	45	4	13.95	14.55	11.57	12.57
8	40	52	6	15.9	17.49	13.63	15.25
9	20	25	8	14.73	18.84	11.92	15.94
10	40	35	2.64	7.59	10.62	6.48	9.03
11	40	35	6	16.12	17.11	13.17	14.69
12	60	45	8	15.8	20.39	12.7	17.63
13	40	35	9.36	14.67	19.78	10.82	16.82
14	20	45	4	11.87	13.93	9.85	11.65
15	20	45	8	15.09	19.16	11.5	16.1
16	40	35	6	16.28	17.34	13.08	15.08
17	60	25	4	12.31	14.25	11.2	12.23
18	40	35	6	16.07	16.86	12.71	14.61
19	60	25	8	15.32	19.91	12.36	17.35
20	20	25	4	10.81	13.71	9.28	11.28

Table 7- Analysis of variance related to optimization of lipid extraction by combining chloroform-methanol solvents

Factor	df	hexane-ethanol		chloroform-methanol		
		F Value	p-value	F Value	p-value	
model	5	72.21	< 0.0001	211.97	< 0.0001	Significant
Time(A)	1	21.73	0.0004	37.99	< 0. 0001	-
Temperature (B)	1	6.52	0.0229	9.11	0.0092	-
Solvent to biomass ration (C)	1	162.90	< 0.0001	967.50	< 0.0001	-
AC	1	2.53	0.1341	2.51	0.1357	-
C^2	1	162.35	< 0.0001	42.75	< 0.0001	-
residuals	14	-	-	-	-	-
Lack of fit	9	5.30	0.0678	2.42	0.1721	insignificant
Pure error	5	-	-	-	-	-
Cor Total	19	-	-	-	-	-

Fig. 1. Photobioreactor for microalgae cultivation

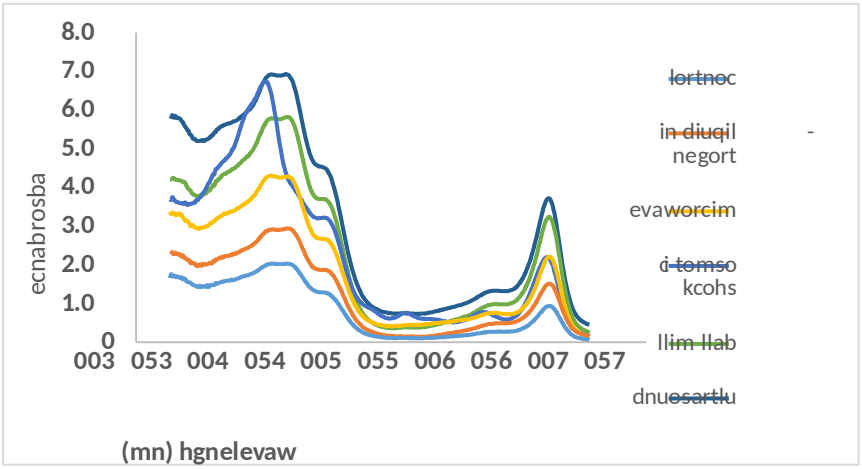

Fig. 2. Spectrophotometric absorption curves of different methods for cell wall disruption

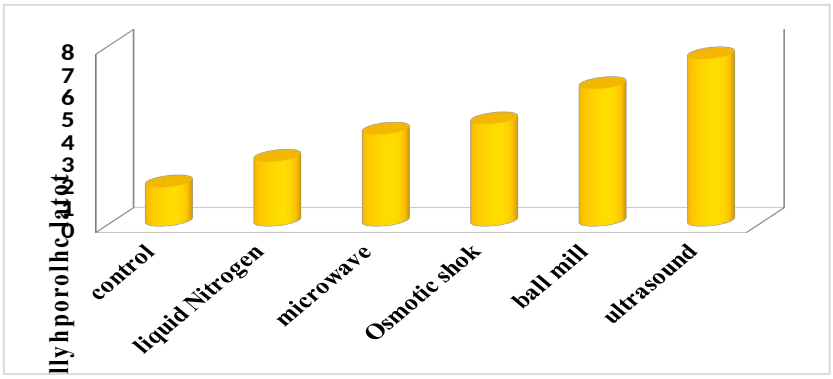

Fig. 3. Comparison of the effect of different cell wall disruption methods on the amount of total extracted chlorophyll from *Chlorella vulgaris*

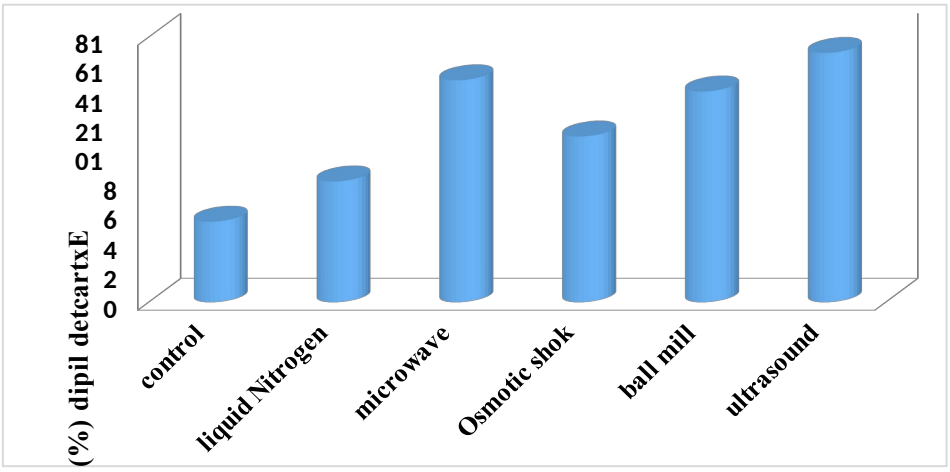

Fig. 4. Comparison of the effect of different cell wall disruption methods on the amount of lipid extracted from *Chlorella vulgaris*

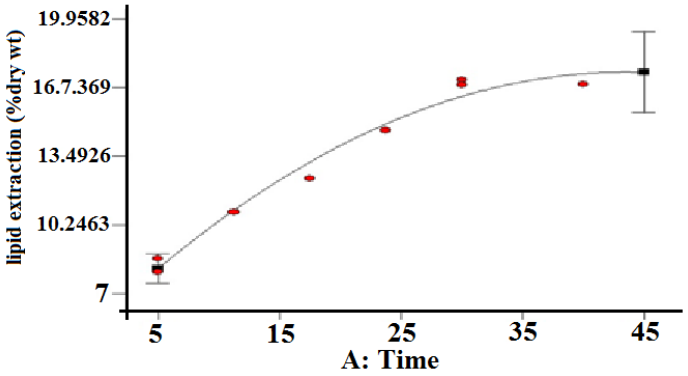

Fig. 5. The effect of ultrasound time on lipid extraction

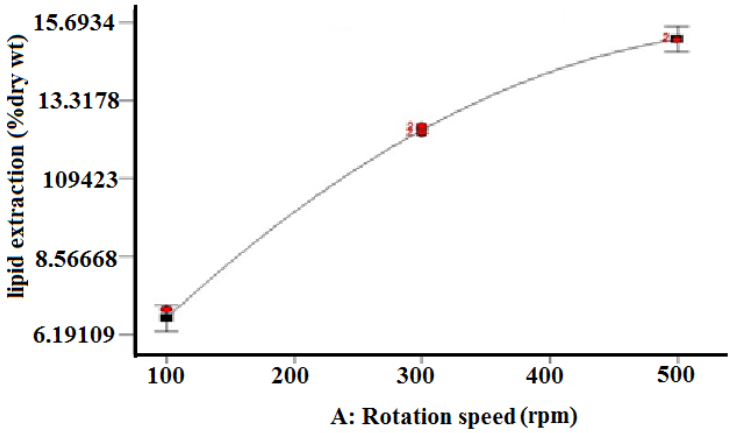

Fig. 6. The effect of milling speed on lipid extraction efficiency

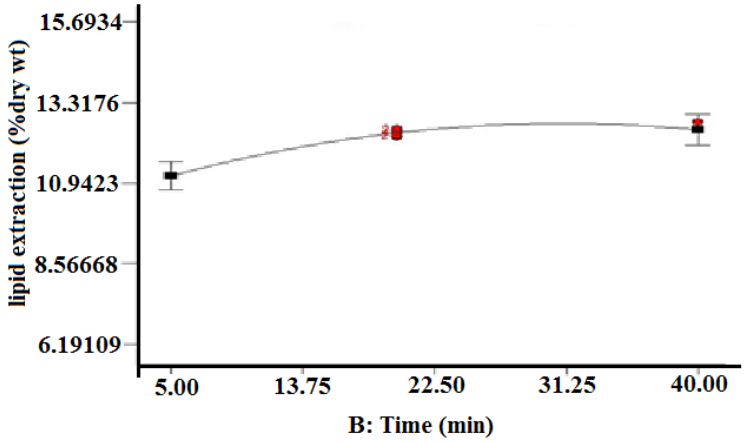

Fig. 7. The effect of milling time on lipid extraction efficiency

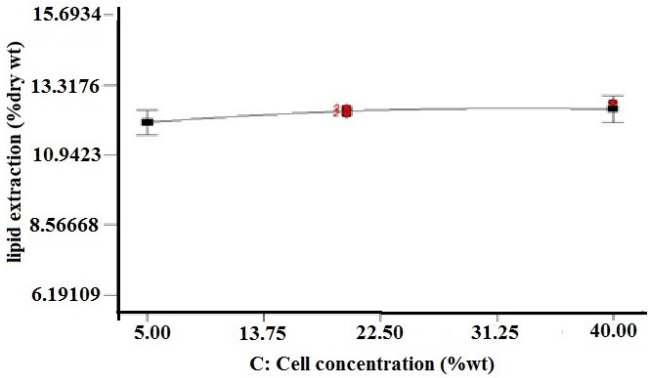

Fig. 8. The effect of cell suspension concentration on lipid extraction efficiency

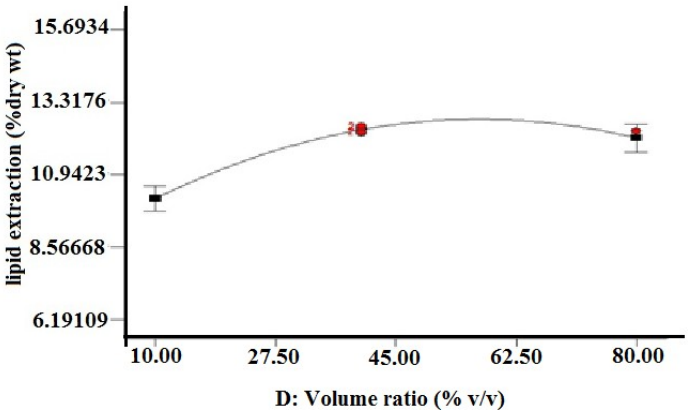

Fig. 9. The effect of pellet volume to cup volume ratio on lipid extraction efficiency

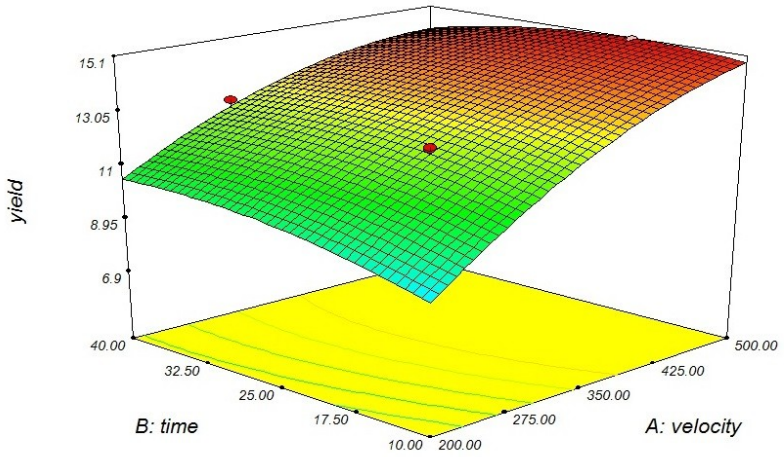

Fig. 10. The effect of time and speed of milling on lipid extraction efficiency

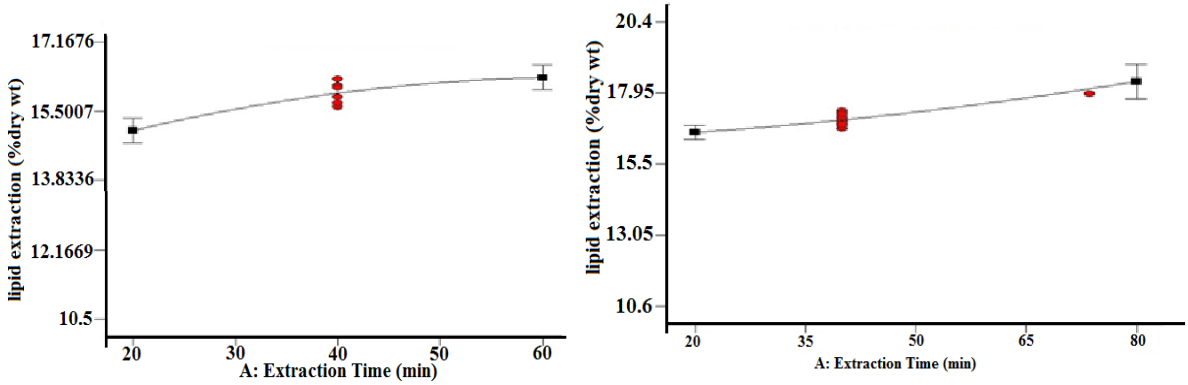

Fig. 11. The effect of time on lipid extraction efficiency by (A) chloroform-methanol solvents, (B) hexane-ethanol solvents

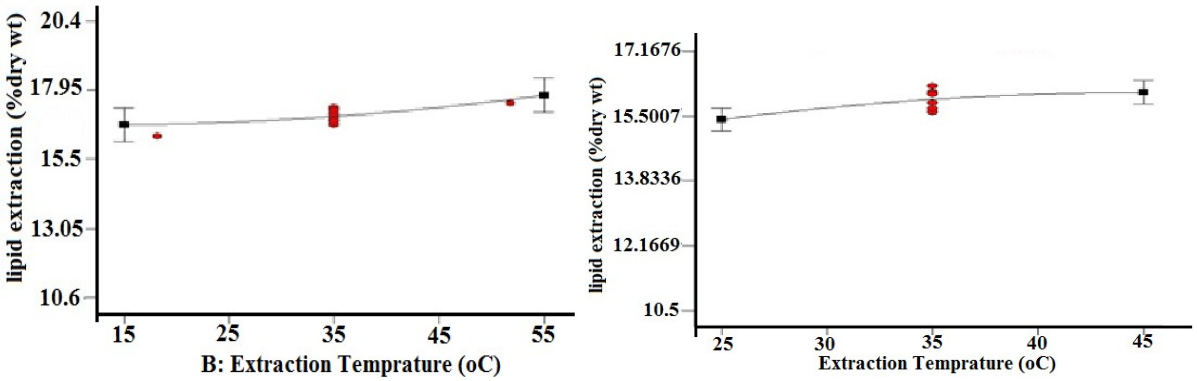

Fig. 12. The effect of temperature on lipid extraction efficiency by (A) chloroform-methanol solvents, (B) hexane-ethanol solvents

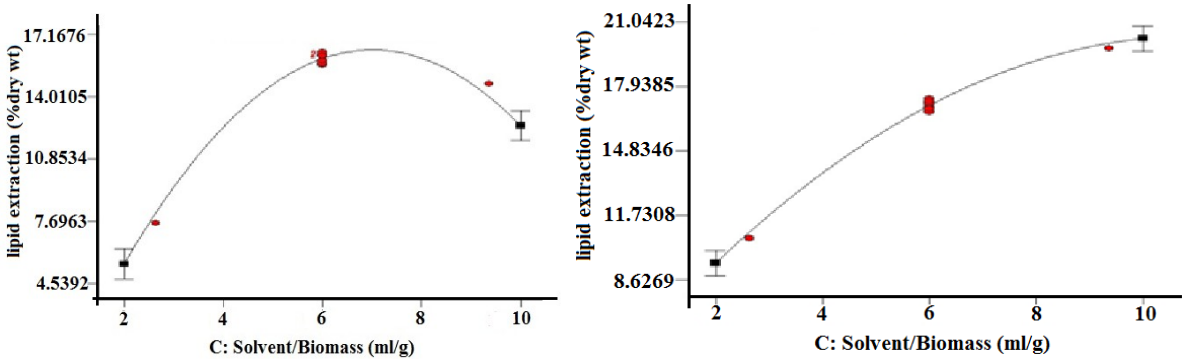

Fig. 13. The effect of solvent to biomass ratio on lipid extraction efficiency with (A) chloroform-methanol solvent, (B) hexane-ethanol solvents

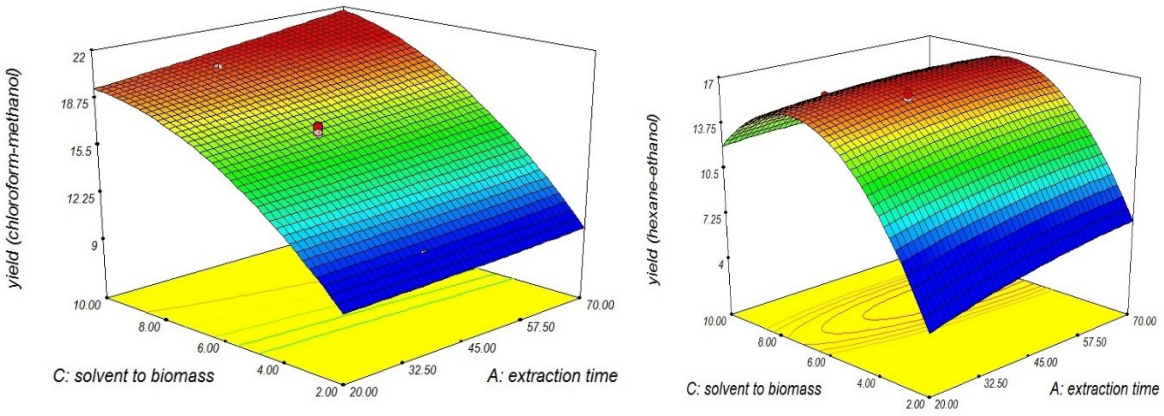

Fig. 14. The effect of solvent on biomass and time on lipid extraction efficiency by (A) chloroform-methanol solvents, (B) hexane-ethanol solvents