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Effect of high pressure homogenization on aqueous phase solvent extraction of lipids from *Nannochloris Oculata* microalgae

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Abstract: The ability to extract lipids from high-moisture *Nannochloris Oculata* algal biomass disrupted with high pressure homogenization was investigated. During the first phase, the effect of high pressure homogenization (system pressure and number of passes) on disrupting aqueous algae (of different concentrations and degree of stress) was investigated. Secondly, the effect of degree of cell wall disruption on the amount of lipids extracted with three solvents, namely: hexane, dichloromethane and chloroform, were compared. Studies reveled that high pressure homogenization is effective on cell disruption while the amount of system pressure being the most significant factor affecting the degree of cell breakage. Although the number of passes had some impact, the level of disruption seemed to level-off after a certain number of passes. The study revealed that slightly polar solvents (such as chloroform and dichloromethane) performed better in aqueous-phase lipid extractions as compared to hexane. Also, it was revealed that it was not necessary to disrupt the algal cells completely to achieve appreciable levels of lipid yields. In fact, conditions that exerted only 20% of the cells to completely disrupt, allowed sufficient damage to liberate most of the lipids contained in the remainder of the cells.

Keywords: Microalgae, Homogenization, Extraction, Lipids

1. Introduction

Using micro-algal biomass constituents as substrates for fuel and chemicals production has attracted wide attention since algae can surpass lipid content and biomass production efficiency of lipogenic terrestrial plants[1-3]. However, there are several challenges that need to be overcome before algal based energy systemsbecome a sustainable reality. One such challenge is the inherentlyhigh moisture of algal growth medium (close to 99.9% w/w – wet basis) that needs to be removed prior to any sort of meaningful processing. Physical and chemical harvesting techniques such as sedimentation, flocculationor centrifugation can only reduce the amount of moisture to a level around 90% (w/w) [4, 5] and further removal of moisture can only be achieved via drying. Drying is energy intensive and removal of such magnitude of moisture is cost prohibitive - especially if reliable solar energy is not available[5-7]. The smaller size of microalgaeof the order of several microns[8]coupled with the presence of an intransigent cell wall [9] thatrequires the cells to be ruptured [10] prior to product extraction, pose additional challenges for processing microalgae. Accordingly, a cell extraction technique, to be successful for algal biomassshould: 1)tolerate high moisture environment and 2) include some sort of cell-wall disruption step to allow the solvent access lipid containing cellular matrixes. This study looks at the effectiveness of extracting algal lipids in a high-moisture environment.

Previously, we have demonstrated that high pressure homogenization can be effectively used for rupturing algal cell walls [11]. In light of this, high pressure homogenization was selected as cell rupture mechanism for this study. Previous studies suggest hexane, chloroform, chloroform/alcohol mixtures, and alcohol to be effective in algal biomass that has approximately < 20% moisture [12-14]. Thus, hexane, chloroform and dichloromethane were used as extraction solvents. This study was directed toward finding the correlation between the degree of cell rupture and the effectiveness of aforementioned lipophilic solvents that are immiscible in water in extracting lipids. Accordingly, a

two phase study was conducted. First, the effect of homogenization on algal cell-wall disruption was studied. Then, the amounts of lipids liberated from algae with various degrees of cell disruption to these solvents were analyzed.

2. Materials and Methods

Algal samples: *Nanochloris oculata* (N. oculata) algal cells (obtained from Texas Agrilife Research Algal Research facility, Pecos, Texas) were used for these studies. The samples contained "stressed algae" that were subjected to physiological stress via nitrogen depletion and those that were not subjected to nutrient depletion. Stressing of algae is known to increase the lipid content [15, 16].

The Total Suspended Solids (TSS) content of an algal broth obtained from a photo bioreactor or an open pond lies around 0.1% (wet weight basis)[5, 17-19]. However, the samples that were used for this study were pre-concentrated at the growth facility to a TSS level of around 17% via centrifugation (for effective shipping).For the lipid extraction technology to be adoptable to a range of harvesting techniques, it was decided to reduce the TSS of algae to 10% via dilution with water. So, all the experiments presented used algae with 10% TSS starting algal concentration (or 90% moisture content wet weight basis) unless noted otherwise.

Homogenization: A NanoDeBEEbench-tophigh pressure homogenizer (Bee international, MA, USA) was used for homogenizing algal samples. The schematic representation of the cross section of the homogenizer is given in the Figure 1.

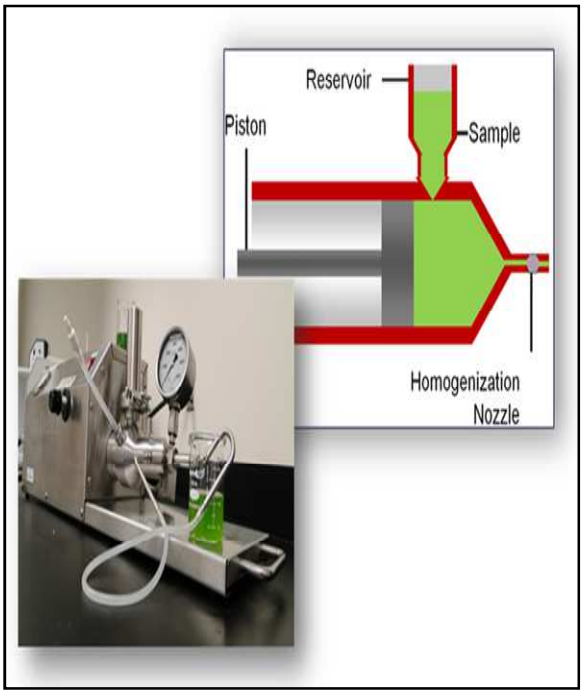


Figure 1. *High pressure homogenizer used for the experiment.*

The homogenizing scheme is as follows: Anaqueous (algal) sample is initially placed in the sample reservoir. A hydraulically operated piston siphons in the sample into the pressure cylinder through a non-return valve. Then, the reciprocating piston forces the sample through a nozzle orifice. As a result of the high shear forces and the sudden pressure drop across the nozzle, algal cells get ruptureddur-ing the passage through the orifice.

2.1. Effect of High Pressure Homogenization on The Degree of Algal Cell Rupture

A study was performed to assess the extent of cell-wall damage on N. oculataby homogenizationunder several variables. The parameters used in the study werepressure, number of passes, stress of the sample and the algal concentration. Levels of these variables are shown in Table 1. A full factorial design was utilized with three replicates at each design point.

Table 1. *List of variables for determining effect of high pressure homogenization on N.oculata.*

Variable	TSS (w/w – wet basis)	Stress	Pressure (Psi)	Number of passes
Levels	0.1% (Non-Concentrated)	Non Stressed	10000	1
	1% (Concentrated)	Stressed	20000	2
			30000	3
			40000	4

Cell Imaging and Counting: Disposable, Neubauer Improved C-Chip DHC-S01 semen counting chambers (Incyto, Chungnam-do, Korea), with 10 µl capacity were used for cell counting.

A Zeiss Axiphot optical microscope with 20x object resolution was used for imaging the cells in counting chambers. A black and white digital camera was used for capturing sections of the counting chamber separately as the entire counting grid was not able to be captured in one image using the required magnification. Image processing software"ImageJ1.42q"(National Institute of Health, USA) was used for counting cells in the images. The degree of cell breakage was characterized as a percentage of the total number of intact cells observed per unit volume. Double counting of cells in multiple images was avoided by cropping images along the gridline.

The intact cell percentage after homogenization (which inversely correlates to the degree of cell breakage) was calculated according to Equation 1:

$$\%Cells\ Remaining = \frac{Cell\ density\ in\ sample\ after\ treatment}{Cell\ density\ in\ sample\ before\ treatment} \times 100\% \quad (1)$$

2.2. Behavior of the Selected Extraction Solvents in Extracting Partially Ruptured Algal Biomass

Solvents Used: Three types of organic solvents were used;namely, hexane, dichloromethane and chloroform. Hexane was selected because it is the industry standard for extraction of lipids in the oleochemical industry. Chloroform was selected due to its slightly higher polarity as compared to hexane and based on prior work on algal oil extraction[11].It was hypothesized that moisture-laden algae would be more miscible/compatible with chloroform due to its slightly polar nature and ability to form hydrogen bonding. Dichloromethane was tested due to its higher miscibility with water, compared to chloroform. It should be noted that hexane and chloroform are considered non-polar solvents while dichloromethane is a polar aprotic solvent and therefore dichloromethane demonstrate a higher miscibility with water compared to chloroform.

Extraction Procedure: Initially, 10 ml of 10 % (TSS) algae (in aqueous environment) were homogenized using a NanoDeBee High pressure homogenizer according to predetermined pressures and number of passes. There pressures and number of passes were selected based on the results from our previous studies[11]to give distinct, percentages of celldisruption. Subsequently, the homogenized algae wasvortexed with 10 ml of solvent for 1 minute at 10, 000 rpm. Then, the mixture was centrifuged at 10,000 rpm for 5 minutes to separate thesolvent phasefrom aqueous phase. Then, 7 ml of solvent waspipetted into an 8 ml high pressure reaction vial (VWR, PA, USA). Lipid exhausted algae and remaining solvent was mixed with an additional 10 ml of solvent and centrifuged at 10 000 rpm for 5 minutes to obtain another extraction. This procedure was repeated to obtain three sequential extractions from a single algal sample. The solvent in the reaction vial was evaporated to obtain lipid-residue.

Transesterification: Transesterification was conducted according to the procedure depicted in Figure 2. First, the residue was dried in an oven at 105^{o}C until the weight of the sample became constant to remove moisture. Then, 4ml of 4% methanolic H_2SO_4 (the transesterification catalyst) and 1 ml of 0.75 mg/ml methanolic C15 Fatty Acid (transesterification internal standard (ISTD)), was added to the dried residue.The purpose of transesterification ISTD is to calculate the transesterification efficiency as described later. This mixture was reacted at 110^{o}Cfor 2 hours while vortexing for 20 seconds at 15 minute intervalsat 10,000 rpm. After 2 hours of reaction, the sample was mixed with 3 ml of hexane and 4 ml of water and vortexed for 1minute at 10,000 rpm to transfer Fatty Acid Methyl Esters (FAMEs) to hexane layer. After vortexing, the sample was centrifuged at 2,500 rpmfor 5 minutes to separate the hexane layer. The hexane layer was pipetted out and filtered using a 0.2 µm PTFE syringe filter. 1 ml of this sample was transferred to a 2 ml GC auto sampler vial and 50 µl of 1mg/ml C13 - C19 fatty acid methyl esters (FAME) in hexane mixture was added as the GC internal standard. This sample evaluated for FAME concentration via GC equipped with a flame ionization detector (FID) (6890 GC, Agilent Technologies, Inc., CA, USA-6850 series with a 30 meter DB-WAX column).

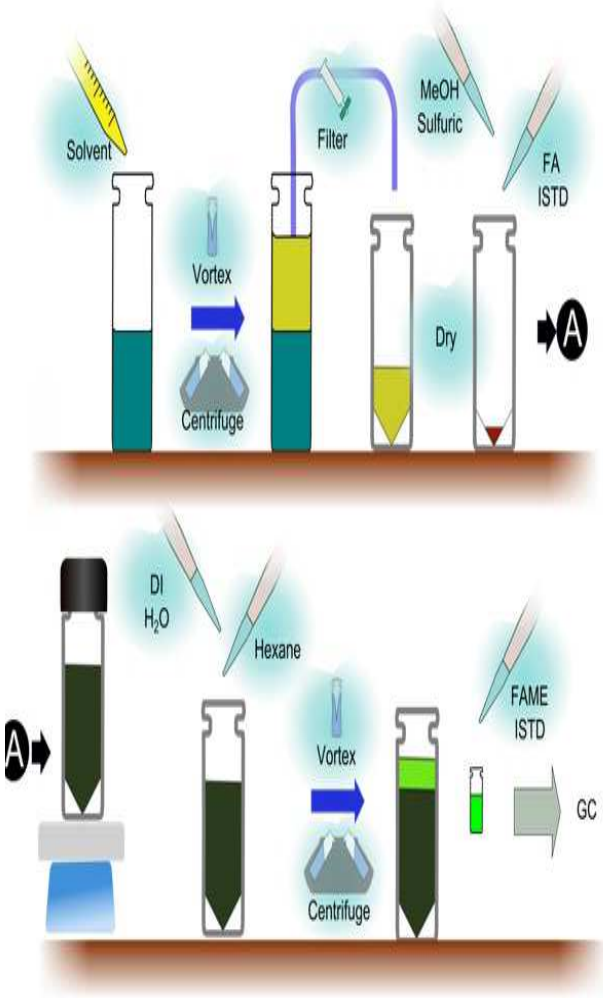


Figure 2. *Extraction methodology.*

In the GC analysis, the area under the signal for each peak of the FID signal was calculated and the resulting area was compared with the signal obtained for the 5% standard FAME mixture(Nu-Chek Prep, Inc. MN, USA - catalog number 68A)in hexane to obtain the total amount of FAMEs in the transesterified sample. Since the amount of C15 fatty acidmixed with the lipid sample and the amount of C15 FAME in transesterified sample is known, it is possible to determine the efficiency of the transesterification. Using the amount of total fatty acid methyl esters and transesterification efficiency, it is possible to calculate the amount of FAs available in the original sample.

Experimental Design: A full factorial design was utilized with extent of cell disruptionand solvent type as independent variables. Table 2shows the entire distribution of the variables used. An asterisk (*) represents a single design point. Three replicates were performed on each design point.

Design Expert 8 statistical analyzing software package

from Stat-Ease, Inc, MN was used for the analysis of results. In this particular segment of studies,a square-root transformation was used to obtain a proper normal distribution.

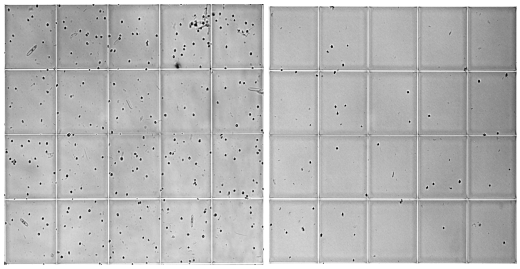
Table 2. *Experimental design for determining the behavior of the selected extraction solvents in extracting partially ruptured algal biomass.*

			Solvent								
			Hexane			Dichloromethane			Chloroform		
Pressure	Number of passes	Cell Disruption	Extraction No.			Extraction No.			Extraction No.		
			1	2	3	1	2	3	1	2	3
N/A	N/A	Non Treated	*	*	*	*	*	*	*	*	*
10 000	2	10%	*	*	*	*	*	*	*	*	*
20 000	2	20%	*	*	*	*	*	*	*	*	*
30 000	3	54%	*	*	*	*	*	*	*	*	*
40 000	4	67%	*	*	*	*	*	*	*	*	*

3. Results and Discussion

3.1. Effect of High Pressure Homogenization on The Degree of Algal Cell Rupture

Two microscopicimages of algal samples before and after homogenization are shown in Figure 3. It is apparent that the number of intact cellshas reduced in the solution matrix (right picture)with increasing pressure treatment (as compared to the left picture). This is because ruptured cell particles are smaller than the resolution selected to image the intact cells.



(a) **(b)**

Figure 3. *(a) Stressed non-homogenized algae; (b) homogenized using Z8 (195 μm) Nozzle, 2 passes, 40 000 PSI (103.421 MPa) (Sample concentration is 0.1%.).*

Results of an analysis of variance (ANOVA) from homogenization studies are depicted in Table 3. According to the analysis, it is clear that all of the variables are statistically significant.

Table 3.*ANOVA table of homogenization of N. Oculata [Classical sum of squares - Type II].*

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob>F)
Block	228.95	2	114.48		
Model	73539.41	8	9192.43	75.55	
A-Concentration	10118.49	1	10118.49	86.16	<0.0001
B-Extentof Stress	551.66	1	551.66	4.53	<0.0001
C-Press	45837.27	3	15279.09	125.58	<0.0001
D-Number of Passes	17032	3	5677.33	46.66	<0.0001
Residual	22022.02	181	121.67		
Corr. Total	95790.38	191			

3.2. Behavior of the Selected Extraction Solvents in Extracting Partially Ruptured Algal Biomass

Statistical analysis was utilized to ascertain whether the variables used, i.e., level of cell disruption and the type of solvent, had any significant impact on the amount of extractable lipids. According to the ANOVA (Table 4), it is clear that all of the variables in fact affected the response variable.

Table 4. *ANOVA table for solvent extraction of algal lipids from homogenized N. oculata.[Classical sum of squares - Type II].*

Source	Sum of Squares	df	Mean Square	F Value	p-value (Prob>F)
Block	0.025	2	0.014		
Model	18.99	8	2.37	103.53	<0.001
A-Solvent	0.73	2	0.37	16.01	<0.001
B-Disruption	4.56	4	1.15	50.09	<0.001
C-Extraction	12.58	2	6.28	273.86	<0.001
Residual	2.48	108	0.023		
Corr. Total	21.49	118			

Diagnostic plots (not illustrated) were utilized to confirm the statistical viability of the experimental design. According to Figure 5, it is clear that the amount of lipids extracted reduced with each extraction iteration (R2 >0.95). The highest amount of lipids extracted was 48.15mg of FA from 1g of algae (Dry basis) with chloroform in the first extraction. In this instance, the third extraction resulted in only 5.7mg of FAs from1g of algal lipids. Although the solvents behaved differently for the initial extraction, they behaved very similar during subsequent extractions.

Figure 4 depicts the variation of percentage of intact cells remaining in the sample after subjecting to various combinations homogenization treatments. It is clear that a higher number of passes and highpressure resulted in a higher level of cell disruption. Although the level of cell disruption seemed to correlate highly with the pressure of the homogenizer, the effect of the number of passes seemed to level off. This may be attributed to physiochemical properties of cell walls that do not respond effectively to repeated exposure of algae to shear (under the same pressure). Nevertheless, the effect of pressure was more profound – algal cells disrupted increasingly with increased pressure. It should be noted that some remaining cell frac-

tion values were higher than 100%. This happens as a result of breakage of the cell conglomerates without significant disruption of cells during low intensity homogenization.

The effects of the degree of cell disruption and the extraction solvent on the lipid yields are depicted in Figure 6. The degree of cell disruption significantly affects the efficiency of lipid extraction. However, the effect tends to level off after about 20% cell disruption. Disrupting cells beyond this level seems not to be necessarily advantageous. This is important in a sense that total disruption of algal cells incurs significantly higher energy costs and is not needed. This tapering effect was more pronounced for hexane as compared to chloroform and dichloromethane. For hexane, the increase of lipid extraction efficiency seemed to level off at as low as 10% cell disruption. It is conjectured that at 10% disruption, most of the cell walls are adequately damaged for the extraction solvents to penetrate into the cell for dissolving lipids. Further homogenization, though disintegrate damaged cells, does not seem to assist in releasing additional lipids. The higher effectiveness of dichloromethane and chloroform, compared to hexane - specially observed in lower percentage cell disruptions may be attributed to higher compatibility of dichloromethane and chloroform with the polar cell wall. It was noted that highly disrupted algae (when at least 67% of the cells are broken), when extracted with any solvent, results in almost 8.5 times more oil than undisrupted algae. However, when the cells were disrupted, the differences between the total amounts of lipids extracted (total accumulation after consecutive extractions) were minimal.

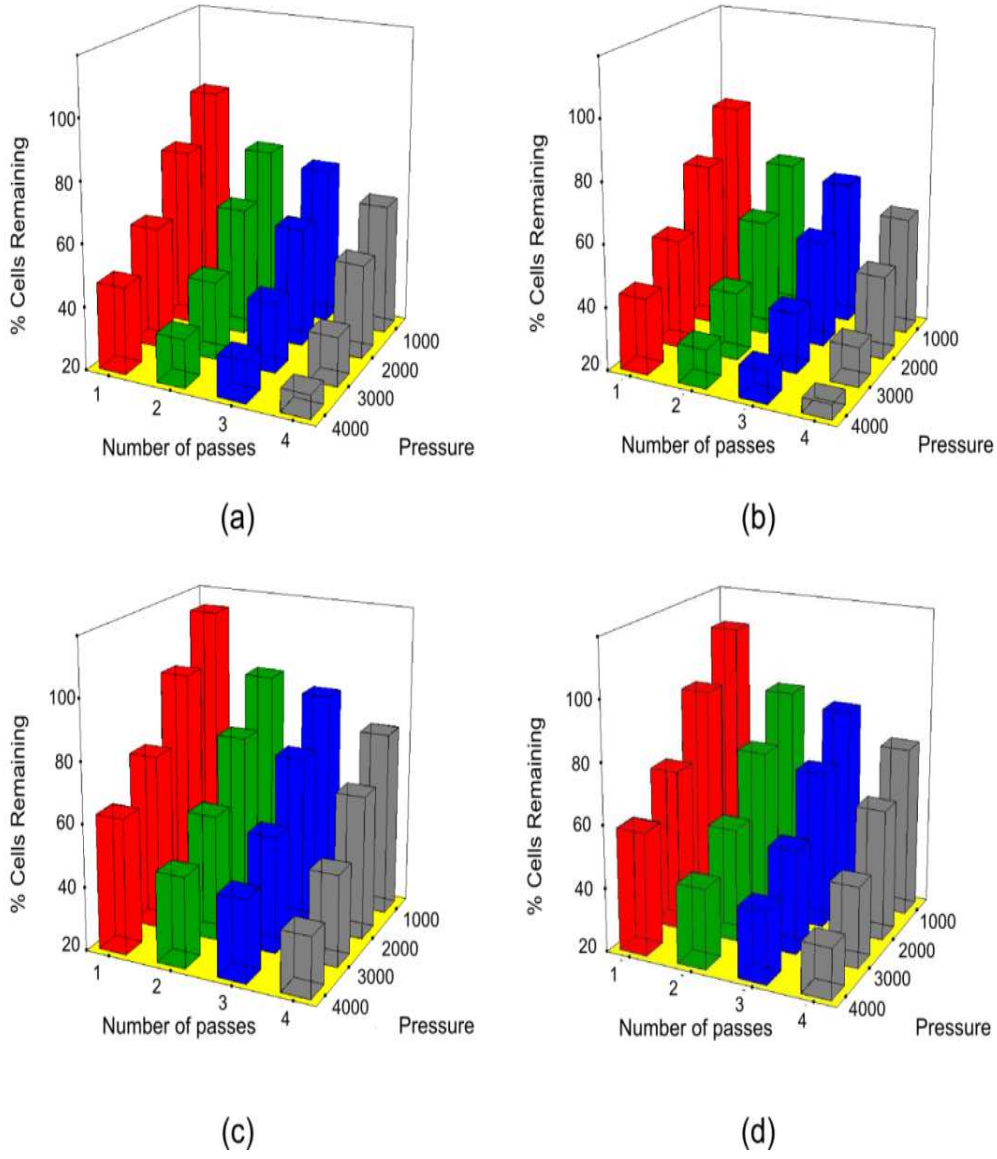


Figure 4. *Percentage of whole cells remaining after homogenization (a) 0.1% concentration, unstressed; (b) 0.1% concentration, stressed; (c)1% concentration, unstressed; (d) 1% concentration, stressed.*

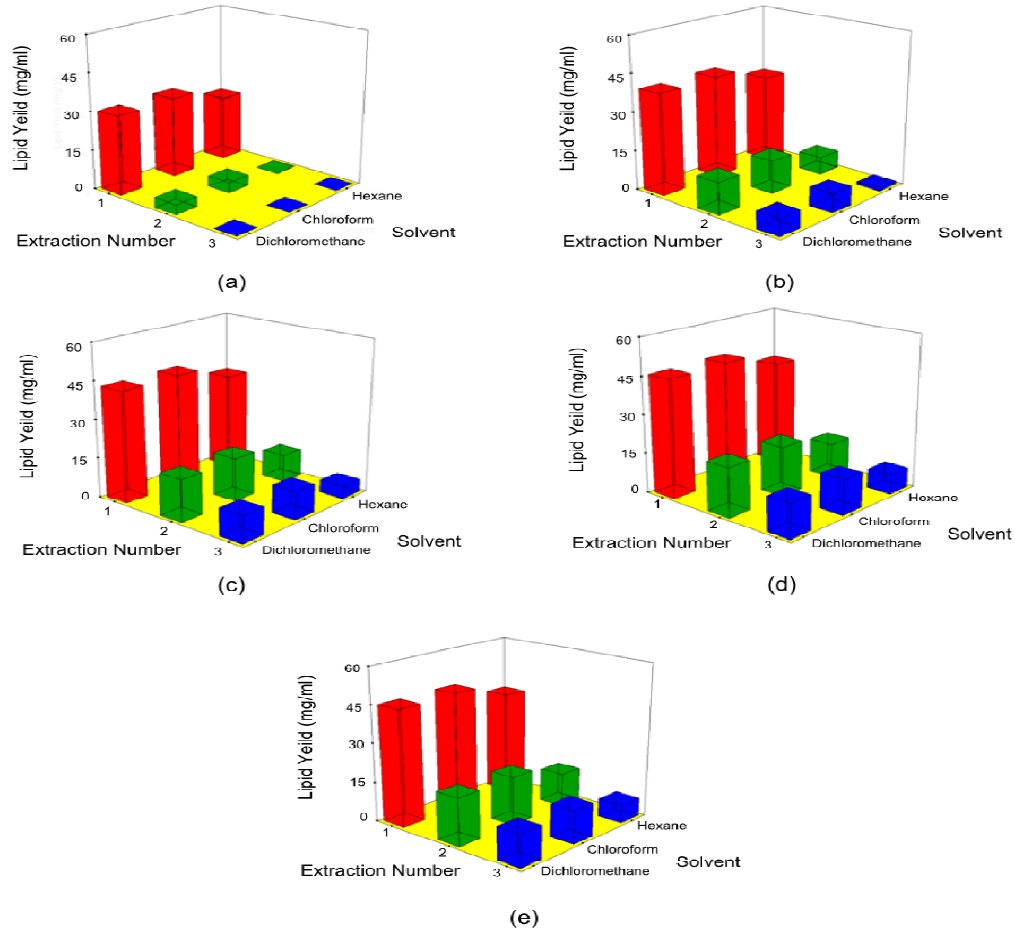


Figure 5. *Variation of amount of lipids extracted when different amounts of cells are disrupted. (a) 0% cell disruption; (b) 10% cell disruption; (c) 20% cell disruption; (d) 54% cell disruption; and (e) 67% cell disruption.*

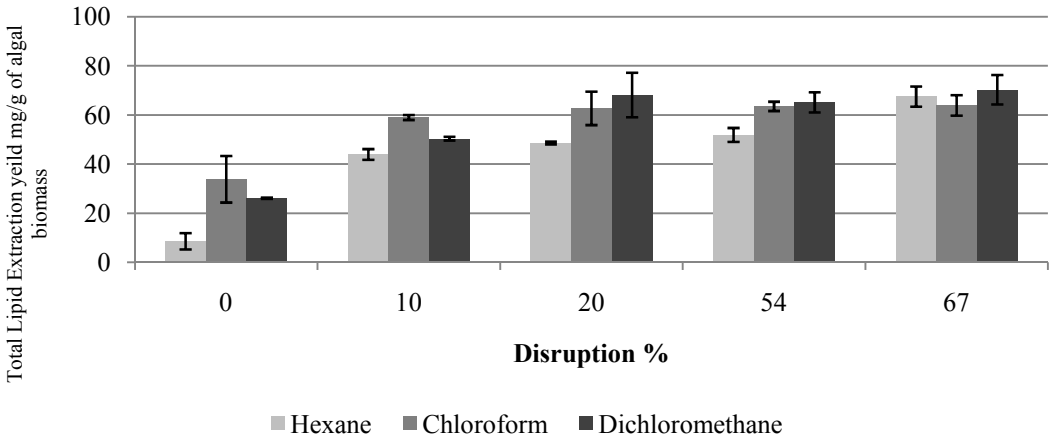


Figure 6. *Lipids extracted from 1 gram of biomass during three consecutive extractions.*

The effects of the degree of cell disruption and the extraction solvent on the lipid yields are depicted in Figure 6. The degree of cell disruption significantly affects the efficiency of lipid extraction. However, the effect tends to level off after about 20% cell disruption. Disrupting cells beyond this level seems not to be necessarily advantageous. This tapering effect was more pronounced for hexane as compared to chloroform and dichloromethane. For hexane, the increase of lipid extraction efficiency seemed to level off at as low as 10% cell disruption. It is conjectured that at 10% disruption, most of the cell walls are adequately damaged for the extraction solvents to penetrate into the cell for dissolving lipids. Further homogenization, though disintegrate damaged cells, does not seem to assist in releasing

additional lipids.

The higher effectiveness of dichloromethane and chloroform, compared to hexane may be attributed to higher compatibility of dichloromethane and chloroform with the polar cell wall. It was noted that highly disrupted algae (when at least 67% of the cells are broken), when extracted with any solvent, results in almost 8.5 times more oil than undisrupted algae. However, when the cells were disrupted, the differences between the total amounts of lipids extracted (total accumulation after consecutive extractions) were minimal.

4. Conclusions

The study confirms that aqueous phase extraction of algal lipids is possible from algal biomass of only 10% TSS (or 90% moisture w/w - wet basis) using hexane chloroform and dichloromethane. It is clear from the analysis that the degree of cell disruption significantly affects the amount of lipids that can be extracted. The study clearly established the fact that although some form of cell disruption is necessary to obtain appreciable oil yields, it is not necessary to totally disintegrate algal cell walls to extract appreciable amount of lipids. Although the amount of lipid extracted increased with increasing level of cell disruption, the increase of lipid yield leveled off after 20% cell disruption.

During the three consecutive extractions, irrespective of the type of solvent used, the second and the third extraction reduced in the amount of lipid yields -approximately to 21% and 11% from the total. Even though chloroform and dichloromethane were more effective in the first extraction, when lipids extracted from all three iterations are considered, hexane trailed behind only slightly. The slight polarity of chloroform and dichloromethane (as compared to hexane) seemed to assist in initial extractions – likely due to the compatibility of these solvents with the polar algal cell walls.

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