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Enhanced Lipid Extraction from Microalgae *Chlorella vulgaris* Biomass: Experiments, Modelling, Optimization

Dmitry Dvoretsky, Stanislav Dvoretsky, Mikhail Temnov, Evgeniy Akulinin, Evgeniya Peshkova

Tambov State Technical University, ul. Sovetskaya 106, Tambov 392000, Russia
dvoretsky@tambov.ru

Microalgae are a promising raw material source for the production of lipids. However, the existing technologies of lipid extraction from microalgae would benefit from their improvement. The objective of the present study was to determine optimal conditions for lipid extraction from *Chlorella vulgaris* (*C. vulgaris*) microalgae biomass.
As a result of the conducted research, a method of cell walls disruption has been selected, and types and ratio of polar and non-polar solvents at the extraction stage have been experimentally identified. Also, the influence of extraction process conditions (temperature, type, and amount of extraction solvents added per unit of biomass) on the degree of lipid extraction from *C. vulgaris IFR №C-111* microalgae biomass has been determined.
The efficiency of microalgae cell walls disruption has been compared for the following disruption methods: microwave radiation, 'osmotic shock', ferromagnetic vortex layer treatment, enzyme solution treatment, and antibiotics treatment.
A comparative analysis of the extraction solvents used for the extraction of lipids allowed to conclude that the use of a mixture of polar and non-polar extraction solvents almost quadruples the recovery of lipids (3.95 times as high) as compared to the extraction with non-polar solvent only. The optimum ratio of solvents of ethanol - petroleum ether 2:1 (vol.), and the temperature of 47÷50 °C provides the maximum degree of lipid recovery – 28 %.
A mathematical model of the lipid extraction process has been developed and optimal conditions for the extraction have been determined.

1. Introduction

Lipid extraction from microalgae is a process that is characterised by high energy consumption. This significantly complicates the use of microalgae biomass as raw material for the production of a wide range of products on an industrial scale (Yang et al., 2014). Two types of processes of lipid extraction from microalgae biomass can be identified: extraction from wet biomass, wherein the extraction of the end product is difficult because of the cell culture fluid, which leads to a low recovery rate in the extraction process, and extraction of lipids from dry microalgae biomass, the drawback of which is high energy costs for drying the biomass.
Thus, with the aim of reducing energy consumption and increasing lipid recovery, the methods of preliminary processing of microalgae biomass have been intensively studied (Table 1).
From the literature review on the subject it can be concluded that no studies have been made to analyse the most effective ways of cell walls disruption and their influence on lipid recovery rate, as well as there is no consensus about what would be the best solvent for the extraction of lipids from biomass. In this regard, the aim of the present study was to determine the best conditions for maximum extraction of lipids from *C. vulgaris* microalgae biomass.
To achieve this goal the following tasks were set and performed: 1) experimental determination of the most effective method of cells disruption of *C. vulgaris IFR №C-111* strain, selection of the best extraction solvent, of solvent – biomass ratio, and temperature of extraction; 2) development of a mathematical model of the process of lipid transition from biomass to the solvent.

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Table 1: Literature review

Study	Biomass type	Best disruption method	Solvent	Lipid recovery, %
Araujo et al. (2012)	*C. vulgaris* dry biomass	ultrasound(40 kHz)	Chloroform – methanol: 1:2 (vol.)	52.0
Prommuak et al. (2011)	*C. vulgaris* lyophilised biomass	ultrasound(40 kHz), microwave radiation (300 W)	Chloroform – methanol 1:2 (vol.)	38.9
Zuorro et al. (2015)	*Nannochloropsis sp.* lyophilised biomass	Enzyme solution (galactomannanase, 1,4 β-cellobiosidase and β-glucosidase)	n-hexane - 2-propanol 3:2 (vol.)	37.3
Concas et al. (2015)	*C. vulgaris* wet biomass	Fenton reaction: 0.5 mole/L H_2O_2 and 0.024 mole/L $FeSO_4$, treatment time up to 3 min.	Ethanol – hexane mixture	17.4
Cho et al. (2013)	*C. vulgaris* biomass of 99 % moisture content	Cellulase at pH=4.8, T= 50 °C, P=72 h	Chloroform – methanol 2:1 (vol.)	10.0
Lee et al. (2010)	*C. vulgaris* dry biomass mixed with distilled water	Autoclave treatment (125 °C at 1.5 MPa for 5 min.), microwave radiation (2450 MHz for 5 min.)	Chloroform – methanol 1:1 (vol.)	10.0

2. Methods and materials

2.1 Cultivation and concentration of biomass

C. vulgaris IFR №C-111 strain was cultivated in the photobioreactor of 2 L volume, with the use of Tamiya OPTIMUM medium, at 30 °C and the illuminance level of 14 kLx for 8 days, before reaching the stationary phase of growth. After that, stress conditions (nitrogen deficit) were created for a period of 6÷7 days (Dvoretsky et al., 2015).

The biomass was concentrated to a moisture content of 95÷98 % using a centrifuge with rotation speed 3000 min^{-1} for 5 minutes.

2.2 Cell walls breakage

Cell walls of *C. vulgaris* microalgae were broken using the following disruption methods: 1) treatment with enzyme solution of CelloLux A and Protosubtilin g3x in 12 mg/mL – 4 mg/mL ratio and exposure time of 10 min. at 55 °C; 2) treatment with amoxicillin solution 0.5 % (mass.) strength; 3) microwave radiation (power 700 W, radiation frequency 280 MHz, treatment time 30 sec.); 4) ferromagnetic vortex layer treatment in electromagnetic field (FVLT) (m (ferromagnetic particles) = 3.85 g, treatment time 15 sec., value of magnetic induction of the rotating electromagnetic field - 0.13 T, the speed of rotation of the field - 30 s^{-1}, magnetic moment $8.635 \cdot 10^{-5}$ $A \cdot m^2$, rotational inertia $0.28 \cdot 10^{-8}$ $kg \cdot m^2$, magnetic field intensity 398.01 A/m, size of ferromagnetic particles l = 12 mm, d = 1 mm); 5) 'osmotic shock' (sodium chloride solution or sucrose solution is added to 15 % (mass.) biomass paste, and after 24 h the mixture is diluted in distilled water in 1:20 ratio).
The determination of the number of intact cells before and after exposure was carried out by direct counting in the Goryaev chamber; the number of cells which lost viability, but retained their shape was counted by adding methylene blue dye to the biomass and directly counting stained cells in the Goryaev chamber. The number of disrupted cells was the difference in the number of cells before and after exposure.

2.3 Lipid extraction

Extraction of lipids from microalgae biomass with disrupted cell walls was carried out in a container with a magnetic stirrer (rotation speed 400 min^{-1}). Estimation of lipids extracted from biomass was done by Zoellner and Kirsch method of determination of total lipids (Zoellner and Kirsch, 1962).
Distillation of the solvent was carried out using a rotary evaporator IR-1 M3 at a temperature of distillation 85 °C and the speed of rotation of the flask 65 min^{-1}.

3. Experimental research into the process of microalgae cell disruption and lipid extraction

For the purpose of selecting an extraction solvent which allows extracting a maximum amount of intracellular lipids from *C. vulgaris* microalgae biomass, an experiment was conducted with the use of non-polar solvents (petroleum solvent C2 80/120, petroleum ether) and polar solvents (ethanol and isopropanol), and mixtures of ethanol and petroleum ether in the ratio 2:1 (vol.) and 1:1 (vol.) as extraction solvents. The results of the experiment are presented in Figure 1. These results allow to conclude that the largest lipid recovery 14.9 % of dry matter of biomass is observed when using a mixture of ethanol – petroleum ether 2:1 (vol.) as a solvent. This result can be explained by the fact that some neutral lipids are located in the cytoplasm not only in the form of lipid globules, but also as complexes with polar lipids. These complexes are attracted by hydrogen bonds to the proteins of the cell membrane. Van der Waals forces arising between non-polar organic solvent and the neutral lipids, which are composed of protein-lipid complexes, are not sufficient to destroy the attraction between lipids and proteins. On the other hand, a polar organic solvent (such as ethanol, isopropanol, etc.) is capable of disrupting the lipid-protein associations by forming hydrogen bonds with the polar lipids in the complex (Kates, 1986). However, along with the neutral lipids which are present in the cells in the form of globules and are included in membrane-associated complexes, polar lipids (phospholipids and glycolipids) are extracted as well.

In order to determine an optimal biomass and extraction solvent ratio for maximum extraction of intracellular lipids from the biomass of *C. vulgaris*, an experiment was conducted with different ratios R of biomass (g) with a residual moisture content of 5 % and a mixture of solvents (ml) (ethanol – petroleum ether in 2:1 (vol.) ratio). The results of the experiment are presented in Figure 2. It can be concluded that the highest lipid recovery rate of 28 % of dry matter of biomass in the extraction was observed when the R ratio of the mixture was 1 (g) of biomass to 200 (ml) of a mixture of solvents. This result can be explained by the fact that a greater amount of solvent allows forming a greater number of Van der Waals interactions (non-polar solvent) and hydrogen bonds (polar solvent) with polar and non-polar lipids; consequently, the formation of more lipid-solvent complexes makes it possible to extract more lipids. Further increase in R leads to a small increment in the amount of the extracted lipids (up to 31 %), but it raises the cost of separation of the solvent by ≈ 8÷10 %.

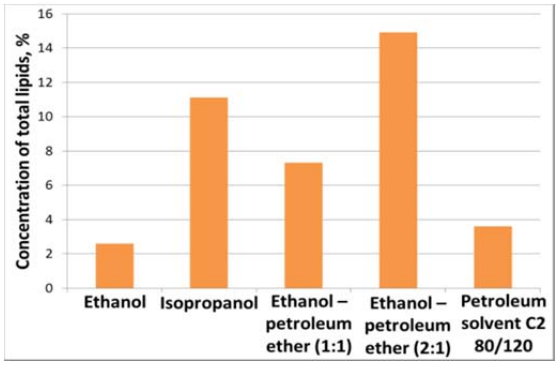

Figure 1: Dependence of lipid recovery on the type of extraction solvent

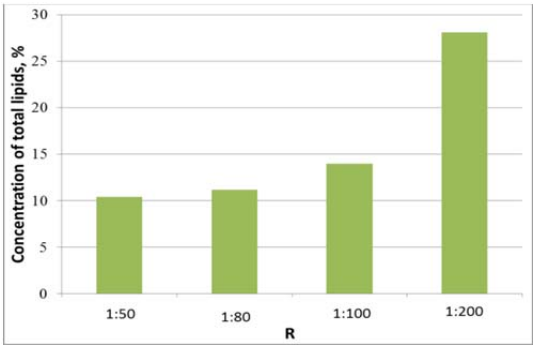

Figure 2: Dependence of lipid recovery on extraction solvent and biomass ratio R

One more experiment was set up to determine extraction temperature which allows extracting a maximum amount of lipids from *C. vulgaris* microalgae biomass. Lipids were extracted with a mixture of ethanol – petroleum ether in 2:1 (vol.) ratio, biomass – extraction solvent ratio R was 1 (g) : 100 (mL), and temperature range 25÷67 °C. The results of the experiment are presented in Figure 3. The largest lipid recovery rate of 26 % of the dry matter of biomass was observed when the extraction temperature was 47 °C, which can be explained by the fact that when the temperature rises above 47 °C, the disruption of heat-labile components of the cell occurs, which leads to a decrease in recovery rate.

With the aim of selecting the best method of breaking cell walls of *C. vulgaris IFR №C-111* microalgae strains, an experiment was performed with the microalgae paste of 98 % moisture content. A cell wall in mature *C. vulgaris* cells has two layers (Burczyk and Hesse, 1981): the first layer is a three layer membrane, which consists of sporopollenin, the second layer consists of mannose and chitin. Such strong cell walls resulted in the fact that, after exposure to a disrupting influence, one part of the cells remained intact (Figure 4, sector *A*), another part of cells lost their viability but retained their shape and were permeable to substances (solvent or dye) from the external environment (Figure 4, sector *B*), and the third part of the cells were disrupted (Figure

4, sector *C*). The results of experiments on the determination of the number of disrupted cells, damaged cells that preserved their structure, and intact cells are presented in Table 2.

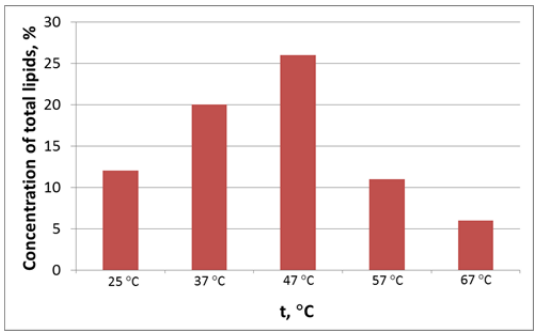

Figure 3. Dependence of lipid recovery on extraction temperature

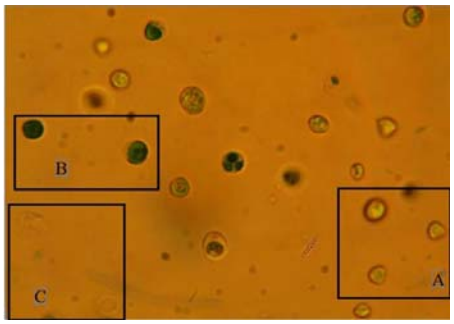

Figure 4: Microscopy of C. vulgaris cells after exposure to a disrupting influence

Based on these measurements, the minimal number of cells remained intact when the biomass was treated with microwave radiation (Table 2), therefore, we can conclude that this is the most effective method of disrupting cell walls of *C. vulgaris*. This is further confirmed by the extraction of lipids from the samples of *C. vulgaris* biomass with cells that have been submitted to different methods of disruption (Table 3).

Table 2: Cell ratio after disruption

Method of disruption	Intact cells, %	Non-viable cells that retained their shape, %	Disrupted cells, %
Antibiotic	88.6	5.4	6.0
Enzymes	81.0	10.0	9.0
Microwave radiation	7.7	43.8	48.5
FVLT	79.0	12.0	9.0
Sodium chloride solution	76.1	17.9	6.0
Sucrose solution	85.5	9.5	5.0

Table 3: Lipid extraction depending on a method of cell disruption

Method of disruption	Lipid recovery, % *Chl. vulgaris IFR № C-111*
Antibiotic	10.0
Enzymes	9.0
Microwave radiation	15.0
FVLT	15.0
'Osmotic shock'	7.0

In order to determine the integrated impact of two methods of disruption on a cell wall during lipid extraction from *C. vulgaris* biomass with 90 % moisture content, an experiment was conducted according to the scheme presented in Table 4. The highest yield of total lipids (22.9 %) is observed when *C. vulgaris* cell walls are broken by integrated effects of 0.5 % antibiotic solution and microwave radiation, and lipids are extracted by petroleum ether and ethanol 1:2 (vol.) solvent mixture.

4. Mathematical modelling of the process of lipid extraction from *C. vulgaris* biomass

The modelling object is the extraction of lipids from *C. vulgaris* biomass, which contains cells of three types (intact, cells that retained the shape but are permeable to the solvent, disrupted, see Figure 4).
Based on the analysis of experimental data it can be concluded that when building a mathematical models the following can be assumed: 1) biomass is a paste with 95 % moisture content; 2) the extractor allows for a perfect mixing; 3) all non-disrupted cells of the biomass have a radius $r \approx 3 \cdot 10^{-6}$ m, surface area S; the cells that lost viability, but retained the shape have radius *r*, the surface area $(S\text{-}S_h)$, where S_h is the area of the holes in the walls of cells (resulting from disruption), and lipid complexes have equivalent diameter

d_e = 4•10^{-9} m; 4) 100 % of lipids is extracted from the disrupted cells, 90 % of lipids is extracted from non-viable cells (I = 0.9 is a coefficient of incomplete extraction of lipids from the cells which lost their viability, while preserving the shape), 70 % of lipids - from non-disrupted cells (J = 0.7 is a coefficient of incomplete extraction of lipids from intact cells); 5) wall thickness of all biomass cells is δ = 10^{-7} m.

Table 4: Lipid extraction under the integrated disruption of biomass cells

Method	*C. vulgaris IFR № C-111*					
NaCl 'osmotic shock'	+	+	-	-	-	-
Sucrose 'osmotic shock'	-	-	+	+	-	-
Antibiotic	-	-	-	-	+	+
FVLT	+	-	+	-	+	-
Microwave radiation	-	+	-	+	-	+
Lipid recovery, %	19.6	20.1	15.3	17.7	17.9	22.9

The extraction of lipids from the cells (intact, disrupted, and retaining its shape, but permeable to the solvent) can be described by mass transfer equation:

$$V \cdot \frac{dC}{dt} = K \cdot F \cdot (C^* - C), \quad (1)$$

where V is the extractor volume, m^3; C^* is the limiting concentration of lipids in the liquid phase (a mixture of biomass and solvent), mole/m^3; C is the current concentration of lipids in the liquid phase, mole/m^3 (a mixture of biomass and solvent), K is mass transfer coefficient, m/s; F is the surface area of contact of phases, m^2.
Depending on the type of cells in the biomass, the terms of Eq(1) are calculated as follows:

1) for intact cells: $C^* = \frac{X \cdot P \cdot N}{1+Z}$, $K = \frac{1}{\left(2 \cdot \frac{r}{n} \cdot D_{\text{int}}\right) + \left(\frac{\delta}{D}\right) + (1/\beta)}$, where X is an accumulated amount of biomass, P is concentration of lipids, N is a share of intact cells, Z is an amount of solvent added, $2r$ is cell diameter, m, n is a coefficient; D_{int} is an internal diffusion coefficient, m^2/s; D is a coefficient of diffusion through a cell wall, m^2/s; δ is thickness of a cell wall, m; and β is mass transfer coefficient, m/s.

2) for non-viable cells which retained their shape: $C^* = \frac{X \cdot P \cdot (1-(N+R))}{1+Z}$, $K = \frac{1}{\left(2 \cdot \frac{r}{n} \cdot D_{\text{int}}\right) + (1/\beta)}$, where R is an amount of disrupted cells.

3) for disrupted cells: $C^* = \frac{X \cdot P \cdot N}{1+Z}$, where the mass transfer coefficient is determined by convection component $K = \beta$.

The surface area of phase contact F were was determined based on the equivalent diameter of the molecules of the protein-lipid complex d_e, taking into account the number of free (unbound by solvent) lipid molecules: $F = \pi \cdot d_e^2 \cdot (C^* - C) \cdot N_a \cdot V$, where N_a is Avogadro's number.

The equations of the model were solved by the Runge-Kutta fourth order method in the Matlab software. The adequacy was checked by comparing calculated and experimental values obtained under the following experimental conditions: $X \cdot P$ = 2.32 mole/m^3; t = 30 °C; w = 2 m/s; N = 0.077; R = 0.485, a mixture of solvents: ethanol - petroleum ether 2:1 (vol.), R = 1:200 (Figure 5). The maximum mismatch was 10 %. From Figure 5 it follows that the implementation of the extraction for longer than 150 minutes is impractical since the bulk of the lipids is already extracted.

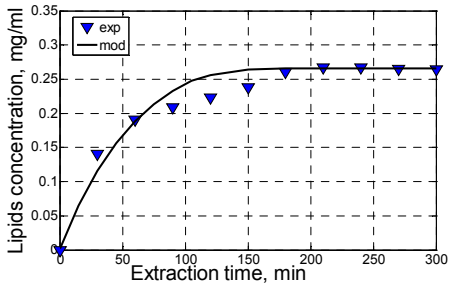

Figure 5: Extraction of lipids from biomass

5. Conclusions

The conducted research has shown that the integrated application of antibiotic solution and microwave radiation to *C. vulgaris* biomass (95 % moisture content) yields the largest lipid recovery rate. The temperature of the biomass during the treatment process must not exceed 50 °C. It has been established that to extract the maximum total lipids a mixture of polar (ethanol) and nonpolar (petroleum ether) solvents in the ratio 2:1 (vol.) should be used. The ratio of the amount of dry biomass (g) to the amount of the mixture of solvents (ml) should be 1:100÷1:200, and extraction temperature – 45÷50 °C. It was experimentally determined that the extraction time during which the major part of lipids is extracted is ≈150 minutes. The mathematical model of the process of lipid transition from biomass to solvent was developed.

Acknowledgments

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