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The Effect of the Complex Processing of Microalgae *Chlorella Vulgaris* on the Intensification of the Lipid Extraction Process

Dmitry Dvoretsky[a], Stanislav Dvoretsky[a], Mikhail Temnov[a], Evgeny Akulinin[a], Antonio Zuorro [b]

[a] Tambov State Technical University, ul. Sovetskaya 106, Tambov 392000, Russia
[b] University of Rome "La Sapienza", Piazzale Aldo Moro 5, Rome 00185, Italy
dvoretsky@tambov.ru

Microalgae are considered a promising source of lipids. However, the existing technologies of their extraction necessitate a massive improvement. In the course of the study optimal parameters of microwave radiation and of enzyme mixture "Cellolux A" and "Protosubtilin G3x" were experimentally selected and theoretically proved so as to allow increasing the yield of lipids 5.75 times to 23 % in comparison with the control sample (5 %). Moreover, the ratio and type of polar and non-polar solvents in the extraction stage for the maximum extraction of lipids was determined taking into account the necessity to process protein-lipid complexes.

1. Introduction

Currently, researchers from around the world focus their attention on the cultivation and processing of microalgae that are considered to be a promising feedstock for the production of renewable energy, food products, animal feed, etc. A growing number of companies are involved in the commercial cultivation and processing of microalgae, increasing the level of development of technologies and performance. However, even the implemented industrial technologies have significant potential for reducing energy intensity and increasing the efficiency of the technological stages (cultivation, deposition, extraction, etc.). One of the production steps which requires lower energy intensity and process time is the extraction of intracellular components. Intensification of the extraction process can be achieved by significantly increasing the contact surface of the cells and solvents and the selection of the optimal composition of the solvents.

There are various approaches that could be used to disrupt the cell walls (Table 1). It can be noted that each of them has its advantages and disadvantages, and recommendations for their use can only be formulated based on the characteristics of specific strains of microalgae and peculiarities of their processing technology.

The object of this study is *C. vulgaris IFR C-111*. In Dvoretsky et al. (2015, 2016) it was found that, under the proper cultivation conditions, the cells can accumulate up to 31 % lipid. However, this species has a tight cell wall which requires special preliminary treatment to facilitate the extraction. As a consequence, the aim of the study was the optimization of the conditions of the stages of microalgae cells' disintegration and extraction of intracellular lipids for the production of ethers of fatty acids from the microalgae biomass.

To achieve this, the following steps were undertaken and analysed: 1) investigation of the mechanisms of disintegration of cell walls and the process of extraction of intracellular lipids; 2) selection of optimal conditions of microalgae cell disruption: with the use of complex enzyme solutions "Cellolux A" and "Protosubtilin G3x" (the exposure time and the ratio of enzymes) and microwave radiation (exposure time and power); 3) selection of a mixture of solvents.

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Table 1: Literature review

Research	Type of biomass	Optimal disruption method and solvent	Lipid yield, %	Lipid yield (control sample), %
Zuorro et al. (2016)	*Nannochloropsis sp.* freeze-dried biomass	Enzyme complex: cellulase 13.8 mg/g, mannase 1.5 mg/g; pH=4.4, T= 53 °C, P=72 hours; n-hexane – isopropanol 3÷2 (vol.)	47	28
Bai et al. (2015)	*C. vulgaris CNW-11*	AES-Bt (*Bacillus thuringiensis* ITRI-G1) 50 mL/g; chloroform - methanol 1÷2 (vol.)	44	34
Pan et al. (2016)	*C. sorokiniana* dry biomass	[BMIM][HSO_4]+microwave radiation (power 800Wt for10-60 min); n-hexane	27	1
Huang et al. (2014)	concentrated algal biomass	Closed reactor with a volume of 1 L which is fed with air with ozone 2 L/min until the pressure reaches 0.6 MPa; hexane – acetic ether 1÷1 (vol.)	27	5 (CBO - ozonizing)
Choi et al. (2014)	Freeze-dried samples of 20 mg of *Chlorella sp.* biomass	[Emim]OAc+[Emim]$(CF_3SO_2)_2N$; methanol-hexane 3÷7 (vol.)	26	19
Orr et al. (2015)	*C. vulgaris UTEX 2714* freeze-dried biomass	[C_2mim][$EtSO_4$]; n-hexane	25	4
Dos Santos et al. (2014)	*C. vulgaris* freeze-dried biomass	Ultrasound 40 kHz for 20 min; Chloroform - methanol 1÷2 (vol.)	19	4
Lee et al. (2010)	*C. vulgaris* dry biomass, mixed with distilled water	Microwave radiation (2450 MHz for 5 min);chloroform - methanol 1÷1 (vol.)	10	5

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 Microalgae cells' disruption

The suspension (100 mL) was treated with microwave radiation at the frequency of 2450 MHz, power 240-700 W. The suspension (100 ml) was also treated with the enzyme complex consisting of two enzymatic solutions: "Cellolux A"; "Protosubtilin G3x" (Sibbiopharm, Derdsk, Russia). The amount of enzyme solutions was calculated on the basis of specifications consumption rates. The disintegration of cell walls was carried out at a temperature of ≈50 °C. In the experiment to determine the effect of the enzyme complex ratio on the output of lipids from the *C. vulgaris IFR C-111* microalgae biomass with 95% humidity content the following ratio of enzyme solutions "Cellolux A" and "Protosubtilin G3x" was used: *sample No.1* – 100 %:0 % (mass.), *sample No.2* is 75 %:25 % (mass.), *sample No.3* – 50 %:50 % (mass.), *sample No.4* – 25 %:75 % (mass.), *sample No.5* – 0 %:100 % (mass.).The experiment to determine the optimal time of exposure to enzymes (the ratio of "Cellolux A" and "Protosubtilin G3x" 75 %:25 % (mass.)) was carried out under the conditions of disintegration of cell walls over the following time periods: *sample No. 6* - 1 minute, *sample No.7* - 5 minutes, *sample No.8* – 10 minutes, *sample No.9* - 15 minutes, and *sample No.10* - 20 minutes.

2.3 Lipid extraction and fatty acid composition analysis

Extraction of lipids from microalgae cells was carried out similarly to the Bligh-Dyer method, with slight modifications: ethanol and petroleum ether in the ratio 2:1 (vol.) over 24 hours at a temperature of 22 °C, with a ratio of dry matter biomass (g) to the amount of solvents mixture (mL) R=1:80. Evaporation of the solvent was carried out using a rotary evaporator IR-1 M3 at a distillation temperature of 85 °C and a speed of rotation of the flask 65 min^{-1}. Estimation of lipids extracted from biomass was done by Zoellner and Kirsch method of determination of total lipids (Zoellner and Kirsch, 1962). Chromatographic analysis was performed in

"Crystallux 4000M" gas chromatograph according to the Russian Federal Standard GOST 30418 “Vegetable Oils. Method for the determination of fatty acid composition".

3. Results and discussion

3.1 Defining the conditions of microwave treatment of cells

Microwave treatment offers several benefits in terms of time and specificity of transformation of electric energy into thermal energy. During microwave treatment an external field constantly changes its direction. As a consequence, the dipoles try to rebuild and start to move, which causes intermolecular friction and increases the temperature of the environment. The effect of the external field creates an oscillating motion of the ions, which leads to their collision with each other and a rapid increase of the temperature. The result is the formation of microscopic local centres of heat, which leads to increase of intracellular pressure and rupture of the cell wall. In the local centres of overheating denaturation of protein molecules is possible, as well as violation of the integrity of cellular and subcellular walls due to hydrophobic interactions breach.

In the course of microwave radiation experiments on the cell wall of microalgae, it was found that the limiting factors are the heating rate, determined by the radiation power, and the average temperature of microalgae biomass after microwave exposure (Figure 1). In these experiments microwave radiation with a power of 280 W was applied to 100 mL of microalgae paste. Samples were stirred for different time intervals from 10 to 50 s with increments of 10 s. The temperature of the microalgae after the treatment ranged from 20 °C up to 95 °C. The highest yield of lipids was observed at the temperature of the microalgae paste after processing of ≈46 °C.

The negative trend of lipid yield with the increase of temperature above ≈46 °C can be explained by the fact that in the process of breaking the cell walls by using microwave radiation the oxidation of lipids is possible. With the increase of temperature active lipid oxidation can be observed: 1) during the destruction of vacuoles and peroxisomes, causing the cell sap containing organic acid to enter the cytoplasm; 2) during the destruction of chlorophyll with the formation of pheophytins. Magnesium ions in the chlorophyll molecule are replaced by two molecules of hydrogen of the cell sap acids, which are admitted into the cytoplasm from vacuoles. The resulting active ions of copper or magnesium are catalysts of oxidation; 3) due to the destruction of heat-labile antioxidants (ascorbic acid, which is destroyed when exposed to ions of copper and iron).

To ensure that the average temperature of the biomass is at the level of ≈46±5 °C the energy should not exceed the value of 6 kJ/mol. The temperature of the microalgae paste can be achieved by using microwave processing of various capacities. The energy with which the microwave generator will energise 1 cm^3 of microalgae paste in 1 second of exposure can be calculated by the following formula:

$$W = (E^2 \cdot \varepsilon \cdot \varepsilon_0 \cdot \omega \cdot tg(\delta))/(4 \cdot \pi), \quad (1)$$

where E is the strength of the microwave radiation field, V/m; ε is relative permittivity of the material (ε =80); ε_0 is absolute permittivity, F/m ($\varepsilon_0 = 8.85 \cdot 10^{-12}\ F/m$); ω is angular frequency of radiation, Hz; $tg(\delta)$ is the tangent of dielectric loss angle for the material substance ($tg(\delta)$ =0.1). Thus, $\omega / 2 \cdot \pi = 2.45 \cdot 10^6\ Hz$, E = 85 (V/cm) at P=280 Watts. Thus, power that will be dissipated in the unit of paste with 98% humidity content will equal:

$$W = (E^2 \cdot \varepsilon \cdot \varepsilon_0 \cdot \omega \cdot tg\delta)/(4 \cdot \pi) = (8500)^2 \cdot 80 \cdot 8.85 \cdot 10^{-12} \cdot 2.45 \cdot 10^9 \cdot 0.1 \cdot 0.5 = 6.3\ \text{W/cm}^3.$$

During the 30 s of microwave radiation treatment 1 cm^3 of the biomass paste will receive the energy 6.3·30=189 J. Let us consider that 1 cm^3 of paste (≈1 g) contains 98% water, 2 % of biomass or n_{water}=0.054 mol, $n_{biomass}$=0.02/24.6=0.001 mol, hence, n_{total} =0.054+0.001=0.055 mol. Then each mole of the paste of microalgae over 30 s of exposure to microwave radiation with a power of 280 W and a frequency of 2450 MHz receives 0.189 kJ/0.055 mol = 3.4 kJ/mol of energy; taking into account heat transfer losses 0.0065 W from the sample to the ambient air, and the heat radiation losses of 0.06 W, the total losses in 1 s will equal 0.067 W and over 30 seconds the losses will be 0.067·30=2.01 J. Thus, 1 cm^3 of the microalgae paste will receive 187.0 J. Considering the losses, during 30 seconds of exposure to microwave radiation with a power of 280 W and a frequency of 2450 MHz, each mole of the microalgae paste receives 0.187 kJ/0.055 mol = 3.39 kJ/mol of energy. The amount of energy that will be transferred to every mole of the microalgae paste under microwave exposure with a capacity of 280÷700 W for 0÷100 s in accordance with calculations by eq. (1) is shown in Figure 2. Therefore, the treatment time of the microalgae paste can vary

from 10 s to 50 s, subject to the treatment temperature, which should also be provided for in the design of microwave disintegrator.

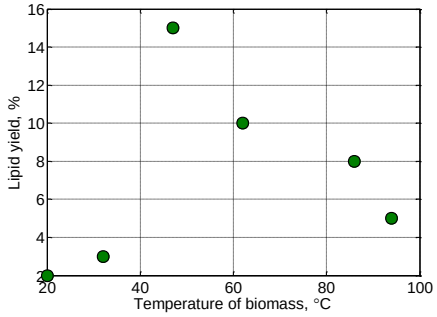

Figure 1: Dependence of the amount of extracted lipids from the microwave treatment temperature

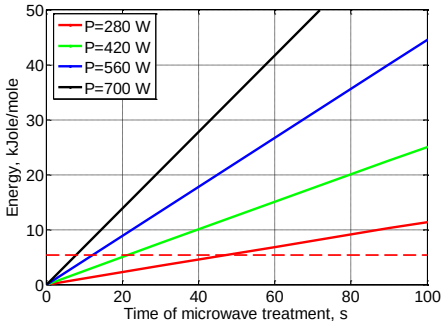

Figure 2: Dependence of the energy absorbed by biomass from the microwave treatment time

3.2 Determining the conditions of enzyme treatment of cells

Analysis of the composition of the cell wall of *C. vulgaris* (Gerken et al., 2012) suggests that the weakening of the strength of the cell wall can be achieved by pre-treatment of cells with complex enzyme solutions consisting of "Cellulose A" and "Protosubtilin G3x". "Cellolux A", containing beta-glucanase, xylanase, cellulase, and alpha-amylase, diffuses to the cell surface and degrades the hemicellulose, which is included in the composition of the cell wall matrix. "Protosubtilin G3x", consisting of neutral and alkaline proteases degrades high molecular weight proteins included in the composition of the phospholipid layer, as well as protein-lipid complexes in the cell cytoplasm.

The highest yield of lipids 9% was observed when the cells were treated with the complex enzymatic solutions "Cellolux A" and "Protosubtilin G3x" (Figure 3 and Figure 4) taken in the ratio of 0.012 mg/mL – 0.004 mg/mL (*sample No.2* - 75 %:25 %) for 10 minutes at a temperature of ≈50 °C.

Analyzing the effect of complex enzyme solutions and microwave radiation it can be assumed that firstly weakening the cell wall by enzymes and, then, creating intracellular pressure due to the heating of the cells can increase the number of disrupted cells. An experiment that implemented the described procedure was conducted under the following conditions: 100 mL of microalgae biomass with a 98% humidity content was treated with complex enzymatic solutions "Cellolux A" and "Protosubtilin G3x", taken in the ratio 75 %:25 % (0.012 mg/mL – 0.004 mg/mL) for 10 minutes at a temperature of ≈50 °C, and then, with the microwave radiation 280 W power for 40 s. Lipids were extracted similarly to the Bligh-Dyer method using ethanol and petroleum ether solvents (2:1 (vol.)).

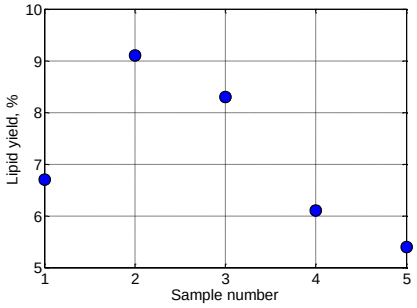

Figure 3: Dependence of the intracellular lipid yield on the ratio of enzymatic solutions

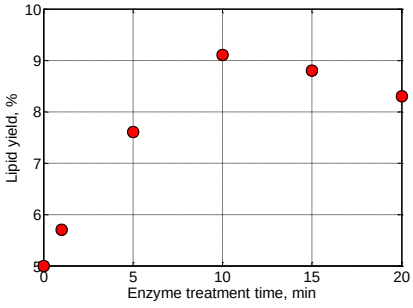

Figure 4: Dependence of the intracellular lipid yield on the duration of enzyme treatment

As a result, the yield of intracellular lipids was 23 %, which is 1.5 times higher as compared with the case of cell disruption using only microwave radiation under optimal conditions, and 2.6 times higher compared with cells disrupted only by enzymes in optimal conditions.

3.3 Solvent selection

In order to select the optimal solvent which can be used to extract the maximum amount of lipids, the following was taken into account: cells may contain lipids in the form of droplets in the cytoplasm of cells and in protein-

lipid complexes, in which proteins and lipids are connected by hydrogen bonds and hydrophobic interactions (Lehninger, 2008). The lipids located in the cell drops represent triacylglycerides – non-polar lipids, the main function of which is the deposition of energy. They can be extracted with non-polar solvent due to the dispersive interactions occurring between the molecules. Cells may also contain lipids in complexes with proteins and polar lipids (Halim et al., 2012), for example in the composition of cell membranes and as soluble cytoplasmic protein-lipid complexes. The lipids included in the composition of protein-lipid complexes can be extracted only in the presence of a polar solvent, since the energy of hydrogen bonds between proteins and lipids is higher than the dispersive interaction between the lipids and non-polar solvent.

In this regard, it is important to determine the ratio of polar and non-polar solvents. It can be done using the Hansen method (Hansen, 2007). It is essential that the selected mixture of solvents allows to extract both triglycerides and the lipids in the protein-lipid complexes. The chromatographic analysis showed that the triacylglycerides (TAG) of *C. vulgaris* microalgae cells are mainly composed of fatty acids: palmitic acid (C16:0) -11.8 %; stearic acid (C18:0) -17.9 % (vol.); oleic acid (C18:1) - 12.5 % (vol.); linoleic acid (C18:2) - 5.8 % (vol.); behenic acid (C22:0) - 5.3 % (vol.); and erucic acid (22:1) - 4.2 % (vol.). Taking into account the chromatographic analysis and certain parameters of solubility for some types of triacylglycerides: triolein (δ_D=16.4 $MPa^{1/2}$; δ_P=3.2 $MPa^{1/2}$; δ_H=4.1 $MPa^{1/2}$), tristearin (δ_D=16.4 $MPa^{1/2}$; δ_P=2.6 $MPa^{1/2}$; δ_H =3.8 $MPa^{1/2}$) and tripalmitin (δ_D=16.3 $MPa^{1/2}$; δ_P=3.1 $MPa^{1/2}$; δ_H=3.8 $MPa^{1/2}$) the solubility parameters of a mean microalgae triacylglyceride (TAG) were calculated, which are the following : δ_D=16.4 $MPa^{1/2}$, δ_P=3 $Mpa^{1/2}$, δ_H=4 $MPa^{1/2}$, R_0=4.7. The solubility parameters of a mean microalgae lipid will be determined based on the following reasoning: microalgae lipids consist of phospholipids and glycolipids (53 %), triglycerides (45 %) and small amounts of free fatty acids (2 %). The parameters of a mean triglyceride have been determined above, and the solubility parameters of phospholipids and glycolipids are described in Hansen (2007). The average solubility parameters of fatty acids will be determined as the arithmetic mean of known solubility parameters: stearic acid, oleic and palmitic acids: δ_D=16.3 $MPa^{1/2}$, δ_P=3.12 $MPa^{1/2}$, δ_H=4.9 $MPa^{1/2}$. Thus, the solubility parameters of a mean lipid will approximately equal: δ_D=16.2 $MPa^{1/2}$, δ_P=3.6 $MPa^{1/2}$, δ_H= 4.14 $MPa^{1/2}$. When determining the solubility parameters of protein-lipid complexes, the solubility parameters of a protein similar in structure to the protein included in the protein-lipid complex were used. For ergosterol the solubility parameters of cholesterol, the animal analogue of ergosterol, were used: δ_D=20.4 $MPa^{1/2}$; δ_P=2.8 $MPa^{1/2}$; δ_H=9.4 $MPa^{1/2}$; R_0=12.6. Taking into account the ratio of substances in a protein-lipid complex (Halim et al., 2012), the solubility parameters will be approximately equal to: δ_D=17.74 $MPa^{1/2}$, δ_P=3.88 $MPa^{1/2}$, δ_H=6.69 $MPa^{1/2}$, R_0=7.1. Ethanol, the most common safe solvent, was selected as a polar solvent. Petroleum ether was used as a non-polar solvent because it has a low boiling point and its evaporation requires the least amount of energy. When using this solvent it is possible to conduct the extraction at a temperature of 45–50 °C (Dvoretsky et al., 2016). The final calculations for the selection of various types of solvents are presented in Table 2.

Table 2: Final calculations of RED for various solvent ratios

Solvent	RED for TAG	RED for a mean lipid molecule	RED for protein-lipid complex	Lipid yield, % (mass) without disruption
Ethanol (E)	3.50	3.43	2.02	2.1
E/PE (4:1 (vol.))	2.61	2.54	1.47	4.0
E/PE (2:1 (vol.))	2.17	2.10	1.21	5.0
E/PE (3:2 (vol.))	1.74	1.67	0.97	8.0
E/PE (1:1 (vol.))	1.33	1.26	0.82	10.0
E/PE (2:3 (vol.))	0.96	0.89	0.75	12.0
E/PE (1:2 (vol.))	0.70	0.65	0.70	12.9
E/PE (1:4 (vol.))	0.69	0.94	0.94	7.0
Petroleum ether (PE)	1.30	1.33	1.40	3.9

▭ - solvent mixture is not suitable ▭ - solvent mixture is suitable

According to the conducted theoretical calculations, the minimum value of the relative energy difference (RED) is observed when all types of microalgae lipids are extracted with a mixture of ethanol/petroleum ether solvents (1:2 (vol.)), which allows to recommend this mixture to be used in the extraction process. The theoretical calculations were further confirmed by experimental research: the use of ethanol / petroleum ether mixture in 1:2 (vol.) ratio allowed to extract the maximum amount of lipids – 12.9 % - from the non-disrupted cells of *C. vulgaris* biomass.

4. Conclusions

Analysis of the mechanisms of influence of microwave radiation on the efficiency of the process of cell walls disruption showed that in order not to induce the oxidation of intracellular lipids the average temperature should be maintained at the level of ≈46±5 °C, in order to avoid the oxidation of intracellular lipids due to local overheating resulting from the irregularity of the electromagnetic field strength and the differences of electromagnetic properties of cell components. Successive treatment with complex enzymatic solutions and microwave radiation has allowed to obtain ≈23 % yield of intracellular lipids, which is ≈1.5 times higher compared to the extraction yield from the cells disrupted by the microwave radiation only, ≈2.6 times higher than the extraction yield from cells that have been disrupted with the use of enzyme complex, and ≈5.75 times higher in comparison with the control sample. A mixture of polar and non-polar solvents, ethanol and petroleum ether, in the ratio 1:2 (vol.), has been selected using the methodology proposed by C. Hansen. This allowed to obtain ≈13% of lipid yield.

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