

## **A method for obtaining plastid pigments from the biomass of *Chlorella* microalgae**

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**Abstract.** Microalgae are distinguished from land plants by the high content of plastid pigments and the biodiversity of carotenoids. The aim of this study is to develop a technology for extracting a pigment complex from the biomass of the microalgae of the genus *Chlorella* and to determine the extracted pigments' composition. To obtain biomass, a crude cell suspension of microalgae was used, which was obtained under laboratory conditions for pre-culture cultivation of *C. sorokiniana* (strain 211-8k). The extraction of plastid pigments from air-dry biomass after disintegration of cell membrane was performed in the 40 kHz mode. It was found that the highest pigment content in ethanol extracts was observed after 30 min ( $870.0 \pm 27.1$  mg L<sup>-1</sup>) at 45–50 °C. The pigments' composition in the resulting total extracts was determined by spectrophotometry and the Reverse Phase HPLC method. The established content of chlorophyll *a* in the obtained extracts was  $537.5 \pm 10.0$  mg L<sup>-1</sup>, the content of chlorophyll *b* was  $182.5 \pm 27.5$  mg L<sup>-1</sup>; the maximum output of the amount of carotenoids in extracts was  $150.0 \pm 10.0$  mg L<sup>-1</sup>. Thus, the main identified forms of carotenoids in extracts from the biomass of microalgae *C. sorokiniana* were xanthophylls: lutein and fucoxanthin (18.6 and 4.7% of the amount of pigment in extract, respectively) and β-carotene (1.8% of the amount of pigment). It is planned to further fractionate the obtained total extracts of the pigment complex to obtain various forms of chlorophylls and carotenoids to study the spectrum of physiological activity of plastid pigments.

**Key words:** *Chlorella* microalgae, pre-culture cultivation, disintegration, extraction, chlorophyll, carotenoids.

### **INTRODUCTION**

To increase the nonspecific resistance of the body, it is advisable to use multicomponent preparations of natural origin, characterized by a balanced composition of biologically active substances that have a normalizing effect on the plastic, energy and informational types of metabolism (Baunthiyal et al., 2017; Nilova, & Malyutenkova, 2018). At the same time, medical practice has a very limited list of pharmacopoeias used as antioxidants, including ascorbic acid, vitamin E and some bioflavonoids (quercetin, rutin, ascorutin, dihydroquercetin) (Nilova et al., 2017).

Algae are a unique source of phytochemical compounds with antioxidant properties (Dymova & Golovko, 2018, McQuistan et al., 2012). Microalgae *Chlorella sorokiniana* is a promising producer of valuable components: proteins (up to 40% dry biomass),

lipids (up to 20% dry biomass), carbohydrates (up to 30% dry biomass) and biologically active substances (Lizzul et al., 2018), which can be obtained by cultivation in laboratory and industrial conditions (Politaeva et al., 2017). The relative content of microalgae components depends on cultivation conditions and age of the population (Sathasivam & Ki, 2018).

The content of plastid pigments, chlorophylls and carotenoids in microalgae can be up to 3.5% in dry biomass, which exceeds their content in land plants (Galasso et al., 2019). The authors (Mishra et al., 2011) noted the antioxidant activity of chlorophyll and its derivatives: chlorins, pheophytins and pyrophytylins (Lanfer-Marquez et al., 2005).

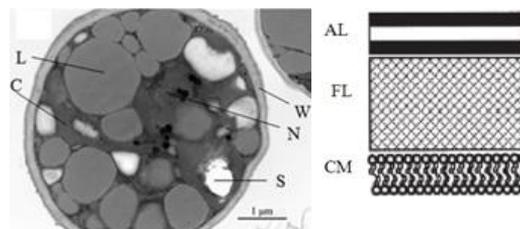
Microalgae are distinguished by biodiversity of carotenoids, their high content (Ambrosino et al., 2019), as well as the possibility of targeted biosynthesis of carotenoids under intensive cultivation of microalgae in the laboratory. It is noted that  $\beta$ -carotene, zeaxanthin, antheroxanthin, violaxanthin, neoxanthin and lutein are characteristic of both microalgae and higher land plants. However, astaxanthin, loroxanthin, fucoxanthin, diadinoxanthin, diatoxanthin, siphonin are found only in the algae biomass (Galasso et al., 2019; Sathasivam 2018).

It is reported that consumption of any available source of carotenoids to significantly reduces photo-oxidative damage to biomembranes, the integrity of cells and tissues initiated by UV radiation (Dymova & Golovko, 2018). In this regard, it is promising to obtain pigment complex concentrates from *Chlorella* biomass in food (Bazarnova et al., 2019a; Kuznetsova, et al., 2019) and pharmaceutical industries.

When extracting pigments from the microalgae biomass, several features should be considered. It is known that photosynthetic pigments are localized in chloroplast thylakoids (Ladygin, 2014) (Fig. 1, a), while the cell membrane of the microalgae *Chlorella* is trilameolar and consists mainly of glycoproteins (Fig. 1, b). Therefore, it is necessary to use methods to increase the availability of valuable components of unicellular algae *Chlorella* (Viera, 2018), due to the processes of cell wall disintegration.

Another problem is the instability of extracted plastid pigments, which are characterized by oxidative degradation under the influence of environmental factors: light, temperature, etc. (Albrecht et al., 2001).

The use of polar solvents is recommended for extraction of the amount of plastid pigments from plant materials: 95.6% ethanol, acetone, 1,4-dioxane, etc. (Dymova & Kuzivanova, 2018). Polar proton solvents (ethanol and acetone) are capable of forming hydrogen bonds and stabilizing ionized chlorophyll particles in biomass extracts. Polar aprotic solvents (1,4-dioxane) retain the ability to dissolve ions, but don't contain acidic hydrogen (Jaffer et al., 2019).



**Figure 1.** Ultrastructure of *C. sorokiniana* cell. a) – the image was obtained using a scanning electron microscope: L – lipid drops; S – starch granules; C – chloroplast; N – nucleolus; W – cell wall (Jiang, 2018); b) – cell membrane scheme (D'Hondt, 2017): AL – alginate-based layer; FL – fibrillar layer; CM – cell membrane.

Ultrasonic extraction has a significant advantage over intensive extraction methods, for example, under the influence of microwaves or autoclaving (Jaeschke et al., 2017), as the recoverable substances are not exposed to high temperatures. Sound chemical reactions (Patel et al., 2018) and the phenomenon of cavitation (Antusheva, 2013) are considered as the main mechanism of the effect of ultrasound on biological objects.

The purpose of the research is to develop technologies for extracting the pigment complex from the biomass of microalgae *Chlorella* and to study the composition of the extracted pigments.

## MATERIALS AND METHODS

The biomass of *C. sorokiniana* (strain 211-8k) was obtained by cultivation in a laboratory bioreactor (Bazarnova et al., 2019b, Politaeva et al., 2017). Universal nutrient medium balanced by micro- and macroelements (Crofcheck et al., 2012) was used. Cultivation temperature – 20–22 °C, daylight illumination – 2,000–2,500 Lux (day-night mode) (Bazarnova et al., 2019c), bubbling mode – 1.5 L h<sup>-1</sup>. The initial concentration of *C. sorokiniana* uterine culture cells was 4.14×10<sup>6</sup> mL<sup>-1</sup> cells.

The biomass was concentrated by autoflocculation (pH 10–11), followed by centrifugation at 6,000 rpm min<sup>-1</sup> for 5 min (Bazarnova et al., 2018). The liquid phase was separated by decantation, the precipitate was dehydrated in air without access of light at a temperature of (20 ± 2) °C. The moisture content in the air-dry biomass was (2.5 ± 0.3) %.

A portion of dehydrated biomass of 0.025 g was poured with 10 mL of solvent and subjected to mechanical disintegration using a high-speed Silent Crusher M homogenizer (IKA® Werke, T25 Basic) at 10,000 rpm min<sup>-1</sup> for 5 min, after which a glass container with homogenate was placed in a water-filled bath WUC-A01H (DAIHAN Scientific, South Korea, power 170 W). The extraction was performed under the conditions of voice-over at a frequency of 40 kHz (constant in the experiment) in the temperature range from 40 to 70 °C for 30 min (Bazarnova et al., 2019d).

When studying the effect of the composition of extracting solvent mixtures on the pigment yield, extractants recommended for extraction of plastid pigments from the phototroph biomass were used: ethanol, acetone, 1,4-dioxane (Amin et al., 2018).

After extraction, the mixture was centrifuged at 3,500 rpm min<sup>-1</sup> for 3 min, the volume of the obtained supernatant was adjusted to 50 mL, and then filtered through a Nylon 66 Membranes 0.45×47 mm filter (SUPELCO) under pressure (105 Pa, VP 18R LabTech pump).

Spectrophotometry of the extracts was carried out in the visible and UV spectral ranges in the wavelength range 350–700 nm with a step of 0.2 on a UV-1240 instrument (Shimadzu corporation Analytical division). Calibration was carried out using 96% ethyl alcohol, a 10 mm cuvette.

The analysis of the total composition of pigments in the extracts was carried out according to the absorption maxima of 470, 649 and 664 nm. The concentrations of chlorophyll *a* (*Cha*), chlorophyll *b* (*Chb*) and carotenoids in extracts were determined according to the methodology (Nayek et al., 2014; Bazarnova et al., 2018).

To separate and identify the composition of chlorophylls and carotenoids in extracts, was used the method of reverse phase high performance liquid chromatography (RP HPLC, Reverse Phase HPLC) on a ZORBAX Original column with reverse phase ODS (C18) Analytical, 4.6 mx 150 mm × 5 μm, 883952–702, sorbent characteristic: specific surface area (Sp) – 350 m<sup>2</sup> g<sup>-1</sup>, pore diameter – 7 nm, particle diameter – 8 nm, particle shape – spherical. The methodology (Gupta et al., 2015) was taken as the basis.

Optimization of the chromatographic separation of pigments requires compatibility between the injection solvent and the mobile phase. For this, the obtained extracts were thickened using a vacuum rotary evaporator (5 kPa, 40 °C) and the dry residue was redissolved in acetone. The resulting mixture was further used for chromatography at a dilution of 5 times.

An autonomous modular device with a simple piston pump for HPLC Varian 03-919000-00 9010 Gradient HPLC Pump DT VAC Case Scratched, Discolored REF VA248 was used as HPLC pump. The speed of passing the eluent is 1 mL min<sup>-1</sup>.

To identify carotenes and xanthophylls in the obtained extracts, standard samples of β-carotene (Merck, Germany) and fucoxanthin (Shanxi Fuhun Biotechnology Co., Ltd.) were used.

Detection was carried out spectrophotometrically (Kratos Spectroflow 783 detector) at wavelengths of 440 nm and 650 nm. Identification and quantitative analysis of the components of the analyzed mixture was carried out according to the retention time and intensity of the analytical signal (height and peak area), followed by recalculation of concentrations according to the formula taking into account dilution, as well as using a computer-based data acquisition and processing system according to the method (Gupta, 2015). The value of the analytical signal for the fractions of identified pigments varied from 0.5 to 3.0.

The optical density of the eluate was measured in a specially designed microcuvette at wavelengths corresponding to the absorption maxima of the analytes under study.

Statistical processing of research results was carried out using the Microsoft Office Excel software and the one-way analysis of variance Analysis of Variance (ANOVA).

The obtained experimental data are presented with the reference to confidence interval calculated using the t-criteria. The confidence probability is 0.95 and statistical significance of the given results is  $p < 0.05$ . The samples were examined in 3-fold repeatability mode.

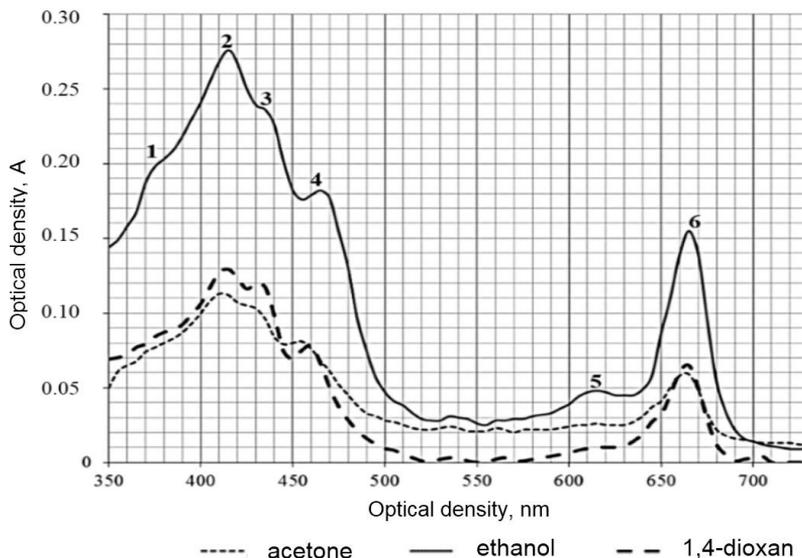
## RESULTS AND DISCUSSION

### Choice of solvent system for extraction of plastid pigments

As a result of the study, it was found that the absorption spectrum of chlorophylls *a* and *b* is characterized by the presence of pronounced maxima: in the red, respectively, 660 and 640 nm and blue-violet, 430 and 450 nm spectral regions (Fig. 2).

The minimum absorption of carotenoids is fixed in the zone of green rays. Carotenes and xanthophylls absorb light only in the blue-violet part of the spectrum. In chlorophyll and carotenoid molecules, a system of conjugated double bonds determines the absorption of blue-violet rays (Borello & Domenici, 2019).

The obtained absorption spectrum of the ethanol extract of *C. sorokiniana* pigments (Fig. 2) is typical for phototroph pigments. The absorption band in the blue-violet region of 380–470 nm has absorption maximums (shoulder 1–380 nm, peak 2–420 nm, shoulder 3–440 nm, peak 4–470 nm), which are characteristic of carotenoids, chlorophylls *a* and *b*. The absorption band in the red region of the spectrum 640–680 includes the maximum (6), which is characteristic of chlorophyll *a* and *b* forms, as well as (5), which corresponds to protochlorophyll (Clayton, 1984).



**Figure 2.** Spectral profiles of total extracts from biomass *C. sorokiniana*.

It was found that under conditions of extraction (40 kHz), ethanol is the most effective as extractant (Table 1) and the most complete extraction of the pigment complex from the biomass of microalgae *C. sorokiniana* is achieved at a temperature of 50 °C (Table 2).

**Table 1.** The effect of extractants on the pigment content in extracts from the biomass of *C. sorokiniana*

Extractants	The pigment content in the extracts, mg L <sup>-1</sup>				
	∑ pigments	<i>Cha</i>	<i>Chb</i>	<i>Cha Chb<sup>-1</sup></i>	<i>Carotinoids</i>
Acetone	517.5 ± 25.0	285.5 ± 20.0	210.0 ± 10.0	1.4	22.5 ± 2.5
1,4-dioxane	607.5 ± 30.0	302.5 ± 15.0	280.0 ± 15.0	1.1	25.0 ± 2.5
Ethanol	870.0 ± 27.1	537.5 ± 10.0	182.5 ± 10.5	2.9	150.0 ± 10.0

Note: in 40 kHz mode, at 50 °C for 30 min.

The maximum yield of the amount of pigments in the extract was (870.0 ± 27.1) mg L<sup>-1</sup>. The maximum yield of chlorophyll *a* – (537.5 ± 10.0) and the amount of carotenoids – (150.0 ± 10.0) mg L<sup>-1</sup>.

An important characteristic of extractants used to work with biological objects is their toxicity. Ethyl alcohol and acetone, according to the safety data sheet, are not classified as acutely toxic, unlike 1,4-dioxane. Ethanol has the least toxicity, which expands the possibilities of its use for pigment extraction in biotechnology of algae biomass processing.

**Table 2.** The effect of the temperature of extraction on the pigment content in extracts from the biomass of *C. sorokiniana*

Extraction temperature, °C	The pigment content in the extracts, mg L <sup>-1</sup>				
	∑ pigments	<i>Cha</i>	<i>Chb</i>	<i>Cha Chb<sup>-1</sup></i>	<i>Carotinoids</i>
40	742.5 ± 37.5	462.5 ± 0.6	145.0 ± 1.3	3.2	135.0 ± 2.5
50	870.0 ± 25.0	537.5 ± 0.4	182.5 ± 1.1	3.0	150.0 ± 7.5
60	827.5 ± 22.5	492.5 ± 0.7	217.5 ± 0.2	2.3	117.5 ± 5.0
70	605.0 ± 27.5	352.5 ± 0.5	170.0 ± 0.5	2.1	82.5 ± 5.0

Note: in 40 kHz mode, for 30 min.

With an increase in the extraction temperature to 60–70 °C, a decrease in the total content of chlorophylls and carotenoids in the obtained extracts is observed, which can be explained by their oxidative degradation (Ötleş, 2016). This process is accompanied by a simultaneous decrease in the ratio of chlorophyll *a* to chlorophyll *b*, indicating the oxidation of chlorophylls and the appearance of their derivatives: pheophytin, pheophorbide, chlorophyllide, chlorin and their oligomers (Antonov & Jagodin, 2006).

In Table 3 presents the results of studies on the effect of the duration of extraction of pigments from air-dry biomass of *C. sorokiniana* with 96% ethanol (40 kHz, 50 °C). It was found that the highest pigment content in the extracts was observed after 30 min.

**Table 3.** The effect of the duration of extraction on the content of pigments in extracts from biomass of *C. sorokiniana*

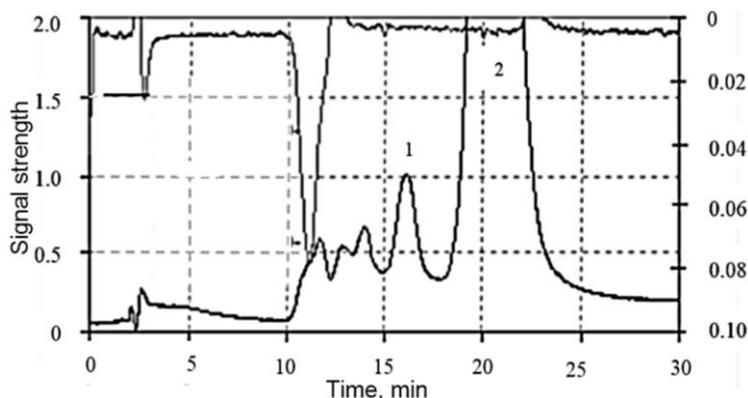
Extraction time, min	The pigment content in the extracts, mg L <sup>-1</sup>			
	∑ pigments	<i>Cha</i>	<i>Chb</i>	<i>Carotinoids</i>
10	597.5 ± 20.0	270.0 ± 12.5	222.5 ± 12.5	105.0 ± 5.0
20	720.0 ± 22.5	335.0 ± 15.0	252.5 ± 12.5	130.0 ± 5.2
30	847.5 ± 27.5	350.0 ± 17.5	385.0 ± 20.0	147.5 ± 7.3
40	847.5 ± 25.0	350.0 ± 17.5	362.5 ± 17.5	137.5 ± 7.3

Note: in 40 kHz mode, at 50 °C.

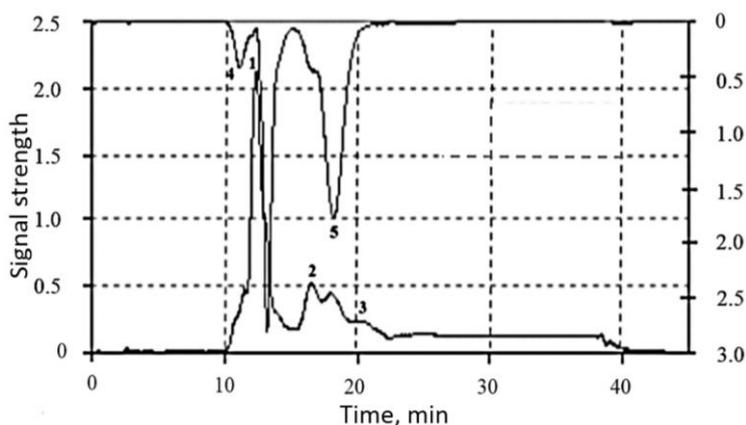
When choosing the mobile phase and the conditions of chromatographic separation of carotenoids and their isomers by the Reverse Phase HPLC method, a gradient mobile phase consisting of methanol, water and acetone was used. The best separation was achieved by the consistent use of a mixture of solvents as an eluent: (A) 0–7 min – methanol : water (75:25); (B) 7–9 min – methanol; (B) 9–19 min – methanol : acetone (80:20); (D) 19–30 min – methanol : acetone (65:35). Maximum selectivity was obtained at a column temperature of 20 °C. The time required for chromatography is 30–40 min.

In Fig. 3 shows a fragment of a chromatogram of carotenoids used as standards.

The results of spectral and chromatographic analysis of the component composition of pigments in extracts of *C. sorokiniana* are presented in Fig. 4 and Table 4.



**Figure 3.** Chromatographic profiles of carotenoids used as standards. Detection at 440 nm – lower curve, left axis; at 650 nm – upper curve, the right axis: 1 – fucoxanthin; 2 –  $\beta$ -carotene.



**Figure 4.** Chromatographic profiles of the main pigments of *C. sorokiniana* (lower curve, left axis, 440 nm; upper curve, right axis, 650 nm): 1 – lutein, 2 – fucoxanthin; 3 –  $\beta$ -carotene; 4 – chlorophyll *b*; 5 – chlorophyll *a*.

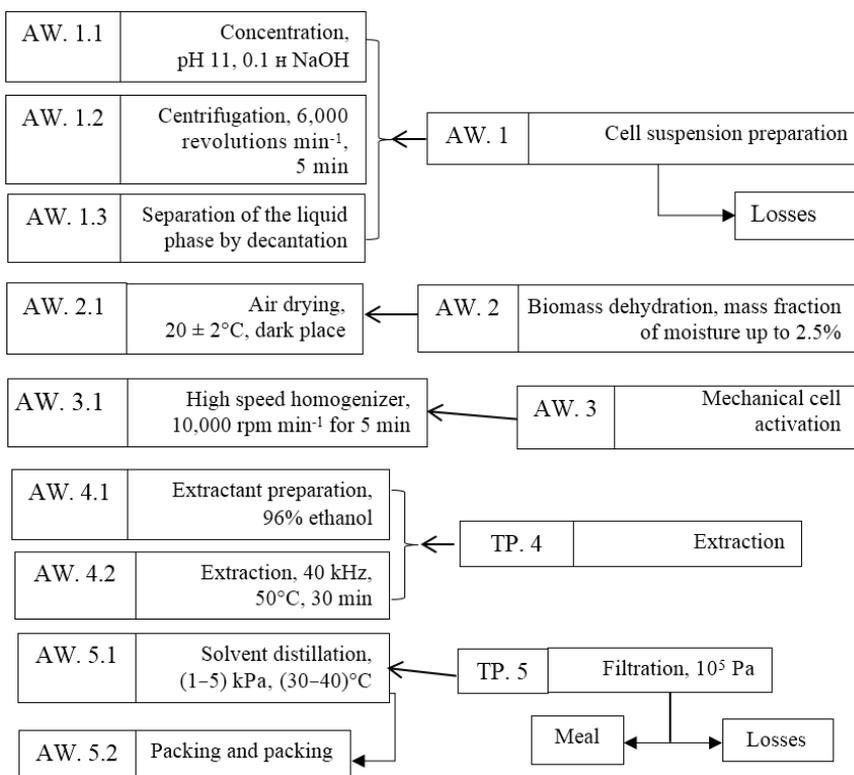
Fucoxanthin, lutein,  $\beta$ -carotene, chlorophyll *a* and *b* were identified by retention times (Fig. 4).

Thus, carotenoids of extracts from the biomass of microalgae *C. sorokiniana* are mainly represented by xanthophylls – lutein and fucoxanthin (18.6 and 4.7% of the amount of pigments in extract, respectively) and  $\beta$ -carotene (1.8% of the amount of pigments).

**Table 4.** The composition of pigments in extracts from biomass *C. sorokiniana*, mg L<sup>-1</sup>

Pigments	Reverse Phase HPLC	Spectroscopy
$\Sigma$ pigments	869.9 $\pm$ 89.3	352.9 $\pm$ 43.3
<i>Cha</i>	270.1 $\pm$ 84.5	199.1 $\pm$ 24.4
<i>Chb</i>	239.2 $\pm$ 28.6	113.1 $\pm$ 13.8
$\Sigma$ carotenoids	–	40.7 $\pm$ 7.1
Lutein	161.6 $\pm$ 14.5	–
Fucoxanthin	40.9 $\pm$ 3.8	–
$\beta$ -carotene	15.7 $\pm$ 1.4	–

Fig. 5 presents the technological scheme of the developed technology for producing pigment extracts from dry biomass of *C. sorokiniana*, including the process of obtaining air-dry biomass, its activation and extraction of pigments.



**Figure 5.** Technological scheme for obtaining extracts of pigments from biomass of microalgae *C. sorokiniana*: AW – Auxiliary work, TP – Technological process.

## CONCLUSIONS

The search for alternative sources of biologically active substances is a promising area of research in biotechnology. The development of technology for the extraction of these substances from plant materials, the determination of their composition and properties is also a key issue (Gomez-Zavaglia et al., 2019). Microalgae of the genus *Chlorella* are considered as alternative sources of carotenoids (Liu et al., 2014).

To extract the amount of pigments from the air-dry biomass of *C. sorokiniana* obtained by cultivation in a laboratory bioreactor, the optimal extraction mode was selected. As the most effective and safe extractant, 96% ethanol is recommended (4 parts by volume of extractant are used for 1 part of dry biomass).

Under the conditions of extraction (40 kHz), the temperature and duration of extraction of pigments from the biomass of microalgae *C. sorokiniana* (50 °C, 30 min) were selected, at which their content in the extracts was  $(870.0 \pm 27.1) \text{ mg L}^{-1}$ , which agrees well with the results obtained by the authors (Villarruel-López A. et al., 2017). The content of chlorophyll *a* in the obtained extracts was  $(537.5 \pm 10.0) \text{ mg L}^{-1}$ ,

chlorophyll *b* –  $(182.5 \pm 10.5) \text{ mg L}^{-1}$ ; the content of the amount of carotenoids –  $(150.0 \pm 10.0) \text{ mg L}^{-1}$ .

Xanthophylls – lutein (18.6%), fucoxanthin (4.7%) and  $\beta$ -carotene (1.8%) of the total pigment content were identified in extracts from the biomass of microalgae *C. sorokiniana* by the Reverse Phase HPLC method. It is good agreement with the data on the carotenoid content obtained by the authors (Matsukawa et al., 2000; Villarruel-López et al., 2017; Lizzul et al., 2018) for microalgae of *C. sorokiniana* cultivated in laboratory conditions. So, for example, as a result of studies (Matsukawa et al., 2000) in the biomass of *C. sorokiniana*, lutein and  $\beta$ -carotene were identified, the content of which was 62% (lutein) and 9% ( $\beta$ -carotene) of the total amount of carotenoids. Researchers (Raman & Mohamad, 2012) report that astaxanthin is present in *C. sorokiniana* (4.0 to 9.5  $\text{mg L}^{-1}$  microalgae suspension).

Thus, a promising area for further study of the obtained total extracts of pigments of microalgae *C. sorokiniana* is their fractionation for the separation of chlorophylls and carotenoids and the study of the spectrum of their physiological activity, whereas this is relevant for their use in the field of biopharmacy (Xu et al., 2017).

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