PROCEDURES OF OBTANING OF EXOPOLYSACCHARIDES PRODUCED BY CYANOBACTERIA *Spirulina (Arthrospira) platensis* AND *Nostoc linckia*

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Abstract The dynamics of accumulation of total and acidic (sulfated) exopolysaccharides (EPS) produced by cyanobacteria *Spirulina platensis* and *Nostoc linckia* under the action of some chemical regulators at the two steps cultivation with the increasing of light at the second step were established. Procedures of EPS isolation from cyanobacteria *Spirulina platensis* and *Nostoc linckia* and the scheme of EPS production, obtaining and quantification have been developed. The synthesis of sulfated exopolysaccharides (till 91 mg/l) at the cultivation of spirulina in two stages was positively influenced by the variation of the light regime and the addition of carbon source with or without addition of Na_2SeO_3. Supplementing of the nutrient medium with sodium selenite (2 mg/l) and varying of light to 2500 lx in the 7th day of cultivation of cyanobacteria *Nostoc linckia* led to a maximum increasing in the amount of total and acidic exopolysaccharides (477 and 422 mg/g, respectively).

Keywords: Cyanobacteria cultivation; *Spirulina platensis*; *Nostoc linckia*; acidic exopolysaccharides; procedures of obtaning.

INTRODUCTION

Exopolimeric substances, including exopolysaccharides, are released by microorganisms (microalgae and cyanobacteria) into the environment during their growth or spread [5, 25]. Many cyanobacteria and microalgae, especially a variety of red algae are manufacturers of various EPS that protect cells under stress conditions. In addition, they are involved in interactions between cells and in the adhesion and formation of the biofilm [6].

Polysaccharides are renewable resources that are assigned to an important class of polymeric materials of biotechnological interest. The characteristics of polysaccharides synthesized by cyanobacteria differ from those of other microorganisms. They have an anionic nature, most of which contain two different uronic acids, a fact specific only for cyanobacteria. Polysaccharides released by cyanobacteria contain one or two pentose residues which are usually absent in other prokaryotic polysaccharides [22]. Most polysaccharides released by cyanobacteria are complex, consisting of six or more monosaccharides. This is the essential difference between cyanobacteria polysaccharides and polymers synthesized by other bacteria or microalgae, where the number of monomers is usually less than four [21].

Due to its chemical, physical and biological properties, polysaccharides offer a wide variety of potentially useful products for humanity. Some extracellular polysaccharides from microalgae and cyanobacteria have different bioactivities that involve anti-tumor, anti-inflammatory and antiviral activity, providing promising prospects for pharmaceutical applications [2, 11].

One review of the results of scientific research on the role and properties of exopolysaccharides in cyanobacteria was carried out by Li et al. (2001) [13]. Cyanobacteria have an increased rate of growth and under the action of stressors they have the ability to proliferate significantly the production of exopolysaccharides (EPS) compared to other microorganisms. More recent information on the production, extraction and characterization of algal and cyanobacterial exopolysaccharides is presented by Delattre et al. (2016) [4].

Exopolysaccharides can be closely bound and remain attached to the cells, or released into the medium and recovered from the supernatant [4, 15]. It is known that soluble EPS can be separated from the culture fluid by precipitation and centrifugation. In the case of EPS attached to the cells outside, an additional treatment is required to remove them. Comte et al. (2007) have established that the selected extraction method depends not only on the content of EPS, but also on their chemical composition [3].

A protocol for the extraction of exopolysaccharides from sludge by physical and chemical methods, which could probably be applied for isolation of exopolysaccharides from microorganisms, including cyanobacteria, was previously developed by Liu and Fang (2002). There were tested some reagents for extraction: EDTA (2% at 4 °C for 3 hours), ion exchange resin (Dowex 50x8, 1 hour), formaldehyde (at 4 °C, 1 hour), formaldehyde (4 °C, 1 hour) followed by NaOH (1 N; 4 °C, 3 h) and formaldehyde plus ultrasonication (60 W for 2.5 min) [14].

Another recently updated protocol was used for the extraction of extrapolymeric substances containing EPS (gel formers) from sludge formed at the biological waste water treatment [8]. In addition to sonication, extraction with solutions of EDTA or formaldehyde with NaOH solutions in the protocol described above, extraction with application of 0.5% Na_2CO_3 at thermal treatment (80 °C, 35 min) was used too. However, the methods used in both mentioned sources are assigned to the extraction of extracellular polymers from the aerobic microorganisms in the sludge and are not adapted for the isolation of EPS in cyanobacteria.

Currently, only few methods of EPS extraction from cyanobacteria including *Sirulina platensis* are known. One method included cultivating of cyanobacteria *Spirulina platensis* for 15 or 30 days and recovering of EPS bound during the linear growth

phase by centrifuging of the culture at 5000 g (10 min at 4°C), followed by suspending of the biomass in 0,05 M TAPS buffer, pH 8.15 with 0.025 M EDTA at 20° and treatment at 100°C (20 min). After removal of the biomass residue by centrifugation, the polysaccharides from the supernatant were precipitated by addition of 3% cetyltrimethyl ammonium bromide and were separated by centrifugation. Classical methods based on centrifugation or filtration of the culture medium did not allow the recovery of EPS from the cell-free liquid medium [9]. The disadvantage of this extraction process is the use of thermal treatment at 100°C, which even for a short duration (20 min) could cause cell degradation and extraction of intracellular polysaccharides.

A selective extraction procedure for polysaccharides from cyanobacteria *Spirulina (Arthrospira) maxima* is also known. Four fractions of polysaccharides were obtained, which included those of: 1) the culture liquid, 2) the outer cell sheath (shell), 3) the cell wall, and 4) the reserve granules (2; 2.6; 10 and 52% of the dry cell mass, respectively) [17].

Recent studies of EPS isolation for *Spirulina* genus has been reported. Thus, in the case of cyanobacteria *Arthrospira platensis* grown for 3 weeks, EPS attached to the cell surface were extracted in two steps. Poorly-bound EPS were obtained by stirring in hot water, then by 0.1 M EDTA were extracted more closely EPS. The fraction of EPS well-soluble in water was found in the culture liquid. This fraction was subjected to membrane filtration (Millipore, 0.45nm) and then to lyophilization [16]. However, the disadvantage of the method is that, together with EPS, the intracellular polysaccharides could be also isolated.

Exopolysaccharides obtaining from the cultural fluid was also described. After separation of the *Arthrospira platensis* culture in the stationary phase, the supernatant was subjected to vacuum filtration, and the EPS precipitation was carried out with 96% alcohol at 4°C. The resulting precipitate was dissolved in water and subjected to ultrafiltration through a membrane filter (cut-off of 100 kDa) at a transmembrane pressure of 4 bars. The concentrated EPS solution was dried at 70 °C [20]. But by this method EPS attached to the cell wall remain no extracted.

Therefore, with reference to the methods of EPS isolation from *Spirulina* genus we can conclude that the research carried out on this subject are quite small, and in some of them have been used thermal processing, which could cause cell lysis and extraction of other intracellular components of biomass . In another research, EPS attached to the cell wall are not isolated separately [24].

Cyanobacterium *Nostoc linckia* is known for their biotechnological potential and as a source of exopolysaccharides. But there are few data on the methods for extraction of the exopolysaccharides produced by azot fixing cyanobacteria. EPS were separated from the strains of *Nostoc* sp. BTA97 and *Anabaena* sp. BTA990 by agitation on a magnetic stirrer for 15 minutes. The cells were macerated and the polysaccharides attached to the cell wall were released into culture liquid. Soluble EPS were separated from intact cells by centrifugation at 6600 g, at 15°C for 20 min. The supernatant was concentrated to one-fourth of the initial volume by evaporation at 60°C for 10-12 hours. EPS from the concentrated liquid was precipitated by the gradual addition of three volumes of cold ethanol and then maintained at 4°C and separated by centrifugation [23]. However, in many cases EPS can not be released in the liquid media and separated by centrifugation.

In the case where the EPS are solubilized in the culture liquid, they can be determined gravimetrically after coagulation with ethanol and drying. Biomass cultivated for 15 days was subjected to homogenization for 5 min. The cellular mass was removed by centrifugation at 10000 g for 10 min. The polysaccharides dissolved in the culture fluid have been precipitated by the addition of 96% alcohol and left for 12 hours at 4 °C. The EPS was filtered, dried to a constant mass and weighed [19]. Thus, by analyzing of the bibliographical sources, we can conclude that in the case of nitrogen fixing cyanobacteria, extraction methods of attached EPS to the cell walls are not developed.

Regarding the EPS isolation from cyanobacteria *Spirulina platensis* and *Nostoc linckia*, we can conclude that the presented methods in this chapter are in a small number and some of them are not adapted to isolate total and acidic exopolysaccharides.

Thus, the goal of this paper was to develop procedures for isolation of exopolysaccharides at the cyanobacteria *Spirulina (Arthrospira) platensis* and *Nostoc linckia*.

The objectives of our research were: selection of the methods and conditions of EPS extraction from cyanobacteria *Spirulina platensis* and *Nostoc linckia*; elaboration of the procedures for EPS amount increasing in cyanobacteria *Spirulina platensis* and *Nostoc linckia*; elaboration of the scheme of EPS production and quantification.

MATERIAL AND METHODS

Cultivation of cyanobacteria Spirulina (Arthrospira) platensis CNM CB-02 was performed in two steps with the addition of carbon source with or without Na_2SeO_3. Samples of 200 ml of cells suspension (0.4 mg / ml) were grown on modified Zarrouk medium at 3500 lx (K, reference sample), and for the other variants, cultivation starting at day 8 was continued at 5500lx. Four experimental variants were examined: two with addition of Na_2SeO_3 (V3 and V4) and two without addition of Na_2SeO_3: V1 only with variation of the light to 5500lx on the 8th day and V2 – with the addition of 2 g of $NaHCO_3$ (as a carbon source). In the case of Na_2SeO_3 addition to the spirulina suspension, 45 mg /l Na_2SeO_3 was supplemented by two ways: portions of 15 mg / l

Na_2SeO_3 in the first 3 days of cultivation (V3) or in the 8th, 9th and 10^{th} day, with the addition of 2 g/l of $NaHCO_3$ at day 8 of cultivation (V4) in both variants. In the 5^{th}, 10^{th}, 15^{th} and 20^{th} days, biomass samples were taken to determine productivity and to extract and determine exopolysaccharides.

Extraction and determination of exopolysaccharides. The spirulina suspension grown 5, 10, 15 and 20 days was filtered to separate the biomass from the culture liquid. After harvesting of the culture fluid, the biomass was suspended in 40 ml of distilled water to extract the exopolysaccharides attached to the surface of the cell wall and after shaking for 4-5 min. Exopolysaccharides extract was filtered with a vacuum pump and the residual biomass was washed with 10 ml of distilled water. The exopolysaccharides fraction was concentrated 10 times in a vacuum evaporator and subjected to dialysis. The concentration of exopolysaccharides was determined by reaction with anthrone sulfuric reagent, and the concentration of acidic polysaccharides - with reagent Alcyan Blue [18].

Cultivation of cyanobacteria Nostoc linckia. Cianobacteria *Nostoc linckia* (Roth) Born. et Flah-CNM-CB-03 was grown by inoculating 0,2 g/l biomass on the Drew nutrient medium. The duration of the cultivation was 14 days, with permanent illumination and 18°C. At 7 days of cultivation the stress factor was triggered. Four experimental variants were examined, of which 3 variants were exposed from 7^{th} day to 2500 lux, with and without the addition of chemical regulators (V2-V4). All variants were carried out in three rehearsals. Option 1 – reference sample without chemical compound cultivated at 1500lx, option 2 (V2) – reference sample without chemical compound cultivated from 7^{th} day at 2500 lx; option 3 (V3) – sample supplemented with 2 mg/l sodium selenite; option 4 (V4) – with 4 mg/l sodium selenite. After cultivation during 14 days, strain productivity was determined by photocolorimetric method and the content of total and acidic exopolysaccharides was established.

Determination of exopolysaccharides produced by Nostoc linckia. Nostoc linckia biomass was homogenized and separated from the culture liquid by centrifugation at 4000 rot/min for 10 minutes. Extraction of exopolysaccharides from demineralized biomass was performed by 0.2 N NaOH , during 12-16 hours at 4°C. After separation of the supernatant by centrifugation for 15 minutes at 4000 g, the amount of exopolysaccharides was determined by reaction with the anthrone reagent. To remove the protein fraction from the EPS solution, the 20% solution of trichloroacetic acid in a ratio of 1: 3 (V: V) was added to the sample. After removing of the precipitate, the sample was dialyzed and acidic exopolysaccharides were determined with Alcyan blue.

RESULTS

Isolation of EPS from cyanobacteria Spirulina platensis. The 200 ml of spirulina suspension was separated from the culture liquid by filtration, and the biomass was washed with 40 ml of distillated water and subjected to filtration by applying a vacuum pump for EPS accumulation in the aqueous extract. After repeated washing of the biomass with another 10 ml of purified water, the extracts were collected together. EPS isolation (previously extracted with water by vacuum filtration) was carried out by precipitation with cold acetone (1: 4, V/V).

Previous studies has shown that the supplementation of the medium with 0.25-0.5M NaCl and $CuSO_4*5H_2O$ (1-4 mg/l) leads to the predominant elimination of EPS in the culture liquid [1]. In the present work in order to increase EPS synthesis in cyanobacteria *Spirulina platensis*, the 2-step cultivation process was used, where as regulators of this process served Na_2SeO_3, the carbon source supplementation and the variation of the lighting regime.

The effect of supplementation of the carbon source ($NaHCO_3$) with and without of Na_2SeO_3 addition, as well as the variation of light from 3500 to 5500 lx at the 2^{nd} stage of cultivation on the spirulina productivity and on accumulation of total and acidic exopolysaccharides (Fig. 1-3) were studied. Productivity values were increased with increasing of cultivation duration, the values were higher, in comparison with the reference sample in all tested variants with the application of physico-chemical factors, regardless of the applied factor (except V3 on 10^{th} day) (Fig. 1).

In order to determine the influence of the light on the production of total and acidic exopolysaccharides (EPS and PSS), their quantity was determined by 2 methods: 1) anthron method and 2) alcian blue coloration. For this purpose EPS were extracted from spirulina samples cultivated for 5, 10, 15 and 20 days as follows: the reference sample (K) – cultivated at 3500lx, and the other samples (V1-V4) grown during 7 days at 3500lx and the rest of the period – at 5500lx with and without addition of chemical regulators.

The analysis of the amount of total exopolysaccharides (determined by anthrone sulfuric reagent) at the addition of the chemical regulators and the variation of the lighting regime (from 3500 to 5500 lx) revealed that compared to the reference sample, their production in the tested variants with and without Na_2SeO_3, reached a maximum increasing of about 1.2-1.4 times on the 20^{th} day, compared to the reference sample cultivated at 3500 lx (Fig.2A).

The sulfated polysaccharides content was increased with increasing of duration of the cultivation (Fig. 2B). Increasing of light intensity on the 8th day (up to 5500 lx) without the addition of chemical regulators (V1) had a low influence on the amount of sulfated polysaccharides determined in the fraction of exopolysaccharides produced by spirulina cultivated

for up to 15 days. After the 15 day period, their concentration was more increased, reaching the maximum value on the 20th day (85 mg/l). A significant amount of produced sulfated polysaccharides was observed until the 15th day in case of addition of Na_2SeO_3 and $NaHCO_3$ at the second stage of cultivation (in the 8th day), but at addition of Na_2SeO_3 in the first 3 days of cultivation the maximum value (of about 100mg/l) were registered in the 20th day.

Similar research on the effect of chemical regulators and the variation of the lighting regime on the productivity and the amount of exopolysaccharides were realized on cyanobacteria *Nostoc linckia.*

Isolation and production of EPS from cyanobacteria Nostoc linckia. For extraction of EPS attached to the cell wall were tested some reagents. Extracting of EPS with H_2O and other reagents such as 0.1M EDTA, 0.5M NaCl did not allow their solubilization. The application of the solutions of 0.1, 0.2, 0.3 and 0.5 N NaOH was also tested, which led to the partial or total solubility of EPS (Table 1).

Analyzing of the data presented in table 1 revealed that extraction of the maximum content of EPS was performed in variants *c* and *d* (262.9 and 263 mg/g, respectively). EPS content was decreased in variants *a* and *b* (176.0 and 210.0 mg / g), related to the insufficient concentration of NaOH solution, the final concentration of which was <0.1N.

The advantage of extraction with 0.2N and 0.3N NaOH solutions was not only the complete extraction of EPS, but also the obtaining of more concentrated EPS extracts and the avoidance of the concentration procedure. After repeated extraction from residual biomass remaining after the first extraction, the presence of EPS was not detected. The EPS extraction with 0.5N NaOH solution was also tested, but in this case partial cell lysis was observed during extraction.

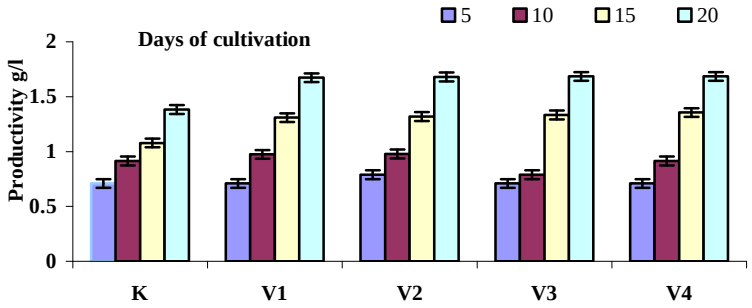

Figure 1. Influence of carbon source supplementation with and without addition of Na_2SeO_3 on productivity at *Spirulina platensis* cultivation in two steps. K – reference sample cultivated at 3500lx; V1 – sample cultivated at 3500 lx first seven days and at 5500lx from 8th to 20th day; V2– sample cultivated with 2g/l of $NaHCO_3$, V3–sample cultivated with Na_2SeO_3 supplemented in the first 3 days and 2 g/l NaHCO3 in the 8th day; V4 – sample cultivated with Na_2SeO_3 supplemented in the 8th, 9th and 10th day and 2g/l NaHCO3 in the 8th day.

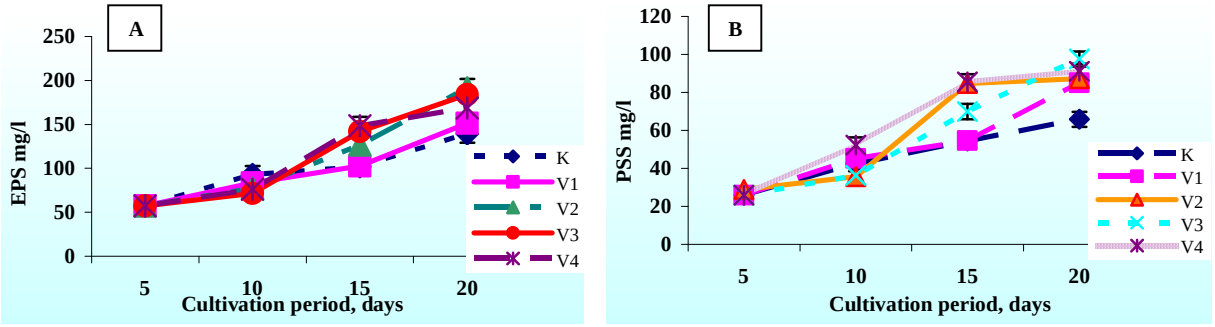

Figure 2. Total (A) and sulfated (B) exopolysaccharides produced by cyanobacteria *Spirulina platensis* under the action of chemical regulators (carbon source with and without addition of Na_2SeO_3) at the two stage cultivation with increasing of light intensity from 3500 to 5500lx at the 8th day.

Table 1. Dependence of quantity of extracted exopolysaccharides attached to the cell wal of *Nostoc linckia* on ratio biomass suspension to NaOH solution (V:V)

Ratio biomass suspension to NaOH solution* (V:V)		Sample of biomass		Quantity of extracted EPS, mg/g
		V, ml	m, mg	
a)	1:7.5	2	9.6	176.0±0.05
b)	1:5	2	9.6	210.0±0.05
c)	1:1	2	9.6	262.9±0.05
d)	2:1	2	9.6	263.0±0.05

This fact may lead to the transfer of intracellular polysaccharides or other intracellular components into the extract.

The next stage of the research was the study of the effect of some chemical regulators and the variation of the lighting regime on the productivity of cyanobacteria *Nostoc linckia* and on the amount of produced exopolysaccharides (Fig. 3A, B). For this purpose two regulating factors were used: 2 and 4 mg/l Na_2SeO_3 supplementation on the 7th day and variation in light from 1500 to 2500 lx.

In the 14th day of cultivation (V1) nostoc productivity was 0.403 g/l. At 2500 lx the cyanobacterial productivity decreased (V2), while the amount of acidic exopolysaccharides was higher than in case of reference sample K (Fig. 3A). It was observed that the most accelerated growth of cyanobacterial productivity (up to 0.546 g/l) occurred at the addition of 2 mg/l of sodium selenite at 2500 lx. In this case, the amount of acidic and total exopolysaccharides also was increased (Fig. 3).

The amount of total exopolysaccharides oscillated between 262-477 mg/g, attesting the maximum value in the sample with the sodium selenite (2mg/l) supplementation in the 7th day and variation in illumination up to 2500 lx (sample V3). In this variant, the maximum values of acidic exopolysaccharides (422 mg/g) were also recorded or 1.7 and 1.3 times more compared to the values of the reference samples V1 and V2 , respectively.

As a result of investigations, the procedures for the cultivation of cyanobacteria *Spirulina platensis* and *Nostoc linckia* for the controlled synthesis of total and acidic exopolysaccharides were developed. An integrated scheme for the production and quantification of EPS in the cyanobacteria *Spirulina platensis* and *Nostoc linckia* (Fig. 4) was elaborated.

The proposed scheme can also be used to obtain exopolysaccharides from other cyanobacteria. In each concrete case the specific conditions and culture medium for the grown of one or the other cyanobacteria, as well as the concentrations of chemical regulators for induction of EPS synthesis, must be established.

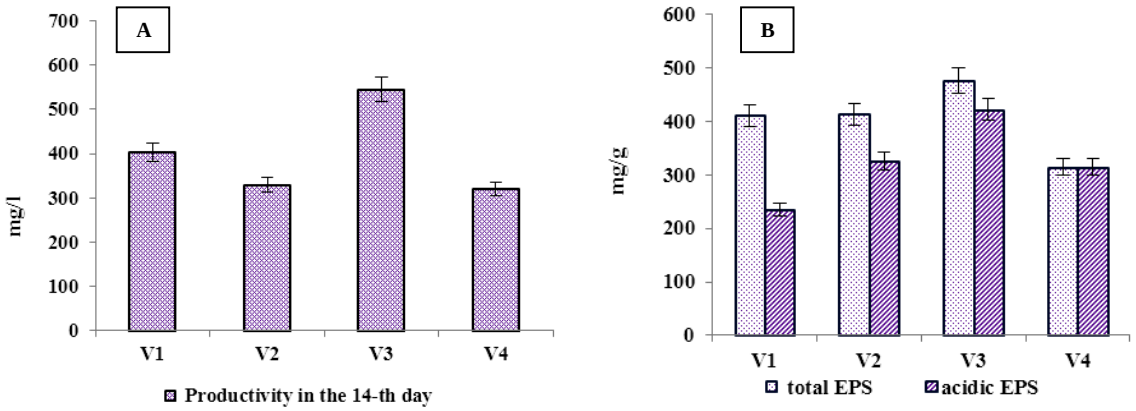

Figure 3. Productivity values of cyanobacteria *Nostoc linckia* (A) and amount of total (total EPS) and acidic (acidic EPS) exopolysaccharides (B) at cultivation on the Drew medium at variation of light intensity and supplementation with sodium selenite; V1- reference sample cultivated at 1500 lx; V2 – reference sample cultivated with variation of lighting regime – 2500 lx in the 7thday); V3 and V4 – samples with supplementation in the 7th day of 2 and 4 mg/l selenite and variation of illumination up to 2500 lx.

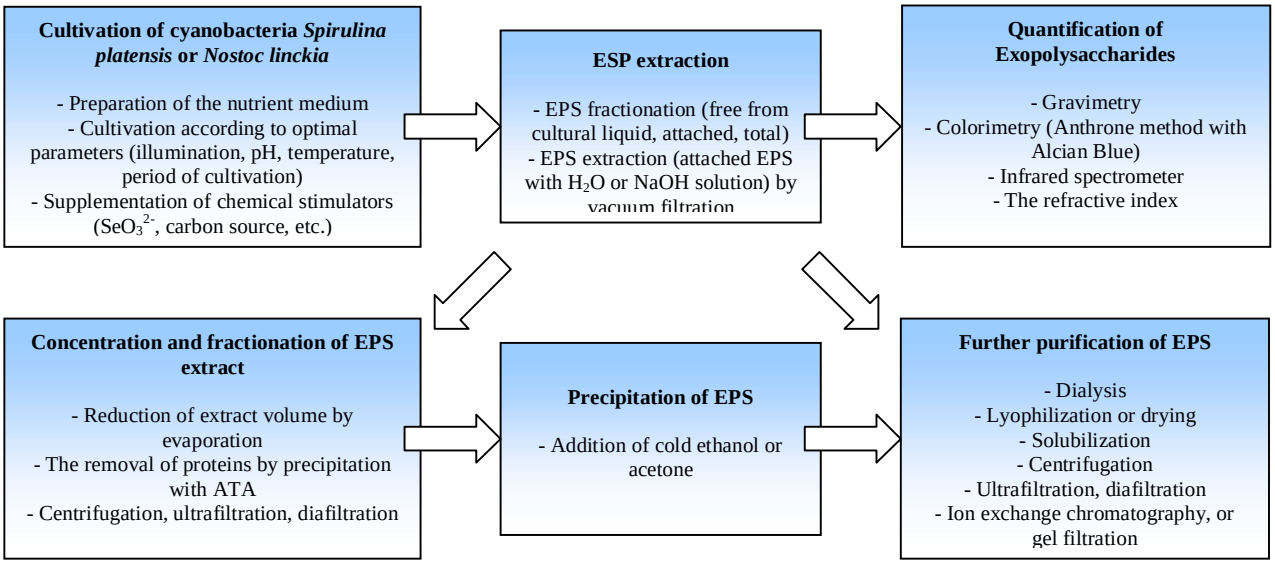

Figure 4. Scheme of production and quantification of EPS in cyanobacteria *Spirulina platensis* and *Nostoc linckia*.

DISCUSSIONS

In general, methods of EPS isolation from microorganisms used by various authors vary from case to case and depend on the chemical nature of polysaccharides, as well as on binding mode to the cell wall. At the same time cyanobacterial EPS can be eliminated in the cultural media or remain attached to the cell membranes. For extraction of attached EPS it is necessary to select the appropriate reagent, which satisfies two conditions: a) to ensure complete extraction of the EPS in a relatively short period of time; and b) the used solvent and extraction conditions (pH, temperature) does not cause damage to cells, because the intracellular polysaccharides and other bioactive substances (proteins, pigments, lipids, etc.) could also be extracted with the EPS.

As a result of investigations, two stage cultivation procedures of cyanobacteria *Spirulina platensis* and *Nostoc linckia* for the controlled synthesis of total and acidic exopolysaccharides were developed. The procedures are based on supplementation of SeO_3^{2-} (or SeO_3^{2-} and carbon source, for spirulina), as well as on increasing of light intensity at the second step of cultivation used as stress factors for EPS synthesis. Previous studies have shown that the supplementation of the medium with 0.25-0.5M NaCl and $CuSO_4*5H_2O$ (1-4 mg/l) leads to the predominant elimination of EPS in the culture liquid [1]. Increased production of extracellular polymeric substances (in our case total and acidic exopolysaccharides) by cyanobacteria can be explained by adaptation towards selenite-induced toxicity. Mechanism of selenite ions influence on cyanobacteria EPS synthesis isn't yet studied. It is known that the cells of *Bacillus* sp. (Strain JS-2) grown in the presence of selenite oxyions secreted large quantities of extracellular polymeric substances. The quantitative and qualitative differences detected in the cell wall fatty acids of the culture grown in the presence of selenite ions revealed the important role of cell wall fatty acids [7].

In previous research carried out by Lee et al. (2012) was proposed two stage cultivation of *Spirulina platensis* for inceasing of polysaccharides production with application at the second stage of cultivation of one of the factors separately: or 0.75M NaCl supplementation, or light intensity increasing from 96 to 192 μmol photons/m^2 [12]. The positive effect of high light intensity on growth and polysaccharide production was established in red and blue-green rhodophyta unicells *Porphyridum* sp. and *P. aerugineum* [10].

The synthesis of sulfated exopolysaccharides (till 100mg/l) at the cultivation of spirulina in two stages was positively influenced by the variation of the light regime and the addition of carbon source with or without addition of Na_2SeO_3 in the 2nd stage. Supplementing of the nutrient medium with sodium selenite (2 mg/l) and varying of light to 2500 lx in the 7^{th} day of cultivation of cyanobacteria *Nostoc linckia* provides a maximum increasing in the amount of total and acidic exopolysaccharides (477 and 422 mg/g, respectively). Such, cianobacterium *Nostoc linckia* can be proposed for acidic EPS production.

The synthesis of the sulfated exopolysaccharides at the two stages cultivation of *Spirulina platensis* was positively influenced by the variation of the light (from 3500 to 5500 lx) and the addition of the carbon source with or without supplementation of Na_2SeO_3 at the first or the second stage of cultivation. The highest values of exopolysaccharides content (85,6mg/l) was registered in the 15th day of cultivation for most cases or in the 20^{th} day (91,1 mg/l) at Na_2SeO_3 supplementation in the first three days of cultivation.

The new procedures of *Spirulina platensis* and *Nostoc linckia* cultivation with Na_2SeO_3 supplementation and variation of illumination at the 2nd stage of cultivation for the directed synthesis of total and acidic exopolysaccharides have been developed.

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