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Porphyridium sp. Microalgae as a source of polysaccharides

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ABSTRACT

Polysaccharides (Ps) are valuable raw materials for a number of industrial sectors. Obtaining Ps from microalgae is both convenient and environmentally friendly. Red microalgae are potential producers of Ps because many of the representatives of this group produce a large number of exo Ps that perform protective functions for cells. This study compared three members of the *Porphyridium* genus for their ability to produce exocarbohydrates in response to nutrient medium chemical composition and lighting conditions. The experiments revealed that strain P-293 compared to P-271 and P-519 produced the highest amount of exocarbohydrates (2.25 g/g on average) under nitrogen source deficiency and sodium chloride excess (0.62 and 18.78 g/L, respectively). Under red-white light conditions, strains P-293 and P-519 produce statistically significantly more exocarbohydrates compared to white and blue light. Strain P-271 produces more exocarbohydrates when cultivated with white light illumination.

1. INTRODUCTION

Marine microalgae are widely used as potential sources of various valuable compounds [1]. Among the various products produced by microalgae, polysaccharides (Ps) are important biomacromolecules [2]. The advantage of microalgae Ps is that it is a renewable material, environmentally friendly, non-toxic and biodegradable, and available at a relatively low cost [3,4]. There are two types of Ps that microalgae produce. Some are stored in the cytoplasm, while others are secreted into the extracellular space and called exopolysaccharides (EPS) [2].

Relatively few studies have been found that provide a comparison of *Porphyridium* sp. species in relation to industrial applications as sources of EPS [5]. Among microalgae used in biotechnological industrial applications, red microalgae belonging to the genera *Porphyridium* and *Rhodella* are gaining interest as sources of valuable compounds [6-8]. *Porphyridium* species are known as sources of sulfated Ps, polyunsaturated fatty acids (arachidonic and eicosapentaenoic acids), phycobiliprotein, phycoerythrin, and as a source of protein [9-12]. Sulfated polysaccharides of red microalgae have attracted increasing attention due to their unique rheological and biological activities [13-15]. These Ps have a high molecular weight (5–7 × 106 Da) [4]. They are thought to have developed through an evolutionary process of adaptation to the effects of salt stress [4].

In the 21st century, interest in bioactive metabolites of marine algae has increased significantly, and many compounds, including microalgae exopolysaccharides, are considered to be promising in terms of their applications in both medicine and aquaculture [16,17]. There are currently numerous works devoted to the biological activity of exopolysaccharides [18,19]. However, they do not lead to significant progress in this field of knowledge because they are predominantly descriptive, are not supported by knowledge of the physiology and biochemistry of specific strains of exopolysaccharide-producing microalgae, and have geographical limitations. In fact, most studies on the applied significance of exopolysaccharides are conducted with two or three Far Eastern (rarely Atlantic) algae species, leaving the richest biological resource of other regions unattended. To move to a qualitatively new level in the study of this matter, a more systematic approach is required. It is necessary to include in the analysis representatives of at least three different strains of the same taxonomic group of microalgae (the most interesting in terms of exopolysaccharide production are *Porphyridium* strains since it is known that they are cultivated under different conditions and synthesize different types of exopolysaccharides [20,21]), to study in detail the structure of molecules included in the extracts of exopolysaccharides of different origins. Implementing research on microalgae exopolysaccharides of various strains will not only contribute significantly to fundamental biology but will also contribute to the development of relevant branches of applied sciences [22].

The study aimed to comparatively investigate the ability to produce exocarbohydrates (exopolysaccharides) of three *Porphyridium* sp. strains (*Porphyridium cruentum* (Ag.) Näg., *Porphyridium sordidum* Geitl., and *Porphyridium* sp. Näg.), depending on the chemical composition of the nutrient medium and type of light. The problem of exopolysaccharide production based on bioprocessing of *Porphyridium* biomass has never been fully solved and continues to be

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relevant due to a lack of a complete understanding of the mechanisms of microalgae biomass accumulation technology, exopolysaccharide production, and the overall cost of the process [23]. This is a scientific novelty, and the practical significance is determined by the possibility of realizing the coproduction of exopolysaccharides and other valuable metabolites (carbohydrates, proteins, lipids, polyunsaturated fatty acids, and pigments), which is of some interest to the pharmaceutical and food industries. Compared to the exopolysaccharides produced by other microalgae, the *Porphyridium* profile offers an alternative source of exopolysaccharides with a high purity index that is classified as food grade [21]. They also find applications in the medical and cosmetic industries as active compounds with antioxidant [22,24], antimicrobial, and anticancer activities. The study's findings will serve as the foundation for technology for growing *Porphyridium* microalgae and producing exopolysaccharides, allowing for the large-scale production of exopolysaccharides with high added value.

2. MATERIALS AND METHODS

The study uses red microalgae of the genus *Porphyridium* (*P. cruentum* (Ag.) Näg., *P. sordidum* Geitl., *Porphyridium* sp. Näg., which were designated as P-271, P-293, and P-519, respectively) from the collection of microalgae and cyanobacteria (K.A. Timiryazev Institute of Plant Physiology of the Russian Academy of Sciences). *Porphyridium* colonies are blood-red or red-brown, incrusting, and have an indeterminate outline. The cells in the colonies are globular. Chloroplast is axial, stellate, with one central pyrenoid. Reproduction is vegetative by simple cell division into two or more daughter cells, sometimes by akinetes. Three microalgae strains were compared due to differences in growth and biomass development rates, cultivation parameters, nutrient medium composition, and accumulation of biologically active substances [21]. The appearance of red microalgae cultures is demonstrated in Figure 1.

Porphyridium cells are spherical or obovate, 5–16 µm in diameter, solitary or grouped in irregularly shaped colonies in a loose mucous matrix, and have no flagella. The cells have no cell wall and are surrounded by a sulfurized polysaccharide complex [4,7-10].

The morphological features of the species *P. cruentum* (Ag.) Näg. (P-271), *P. sordidum* Geitl. (P-293), and *Porphyridium* sp. Näg. (P-519) were described. Cultures of all presented microalgae in glass tubes have a rich red-pink color and demonstrated characteristic adhesion or fouling of the flask walls by the cells [Figure 1]. Despite the external similarity of cultures, the morphology and color of cells differ. Thus, P-271 cells are spherical, light pink, and surrounded by polysaccharide matrix [Figure 1a]. P-293 cells are obovate and bright pink-purple in color [Figure 1b]. P-519 culture cells are spherical in shape, with distinct cell borders and a stained pyrenoid in the center [Figure 1c].

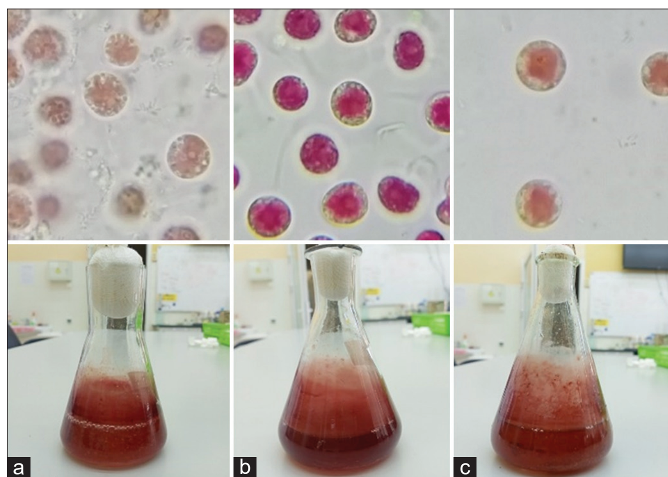

Figure 1: (a-c) Appearance and morphology of *Porphyridium* sp. (from left to right): *Porphyridium cruentum* P- 271, *Porphyridium sordidum* P-293, *P.* sp. Nag P-519, magnification × 1000.

Microalgae were grown on Brody and Emerson nutrient medium max1-2 [25], which contains sources of nitrogen, phosphorus, sulfur, macro-, and microelements. Composition of nutrient medium (g/L): KNO_3 – 1.24; $K_2HPO_4 \times 3H_2O$ – 0.655; KCl – 16.04; NaCl – 12.52; KI – 0.05; KBr – 0.05; $MgSO_4 \times 7H2O$ – 2.5; $Ca(NO_3)2 \times 4H_2O$ – 0.25; $FeSO_4 \times 7H_2O$ – 0.0249. Micronutrient solution (g/L): H_3BO_3– 2.86; $MnCl_2 \times 4H_2O$–1.81; $ZnSO_4 \times 7H_2O$ – 0.22; $CuSO_4 \times 5H_2O$ – 0.08; MoO_3 – 0.015; NH_4VO_3 – 0.023; $K_2Cr_2(SO_4)_4 \times 24H_2O$ – 0.096; $NiSO_4 \times 7H_2O$ – 0.048; $Na_2WO_4 \times 2H_2O$ – 0.018; $Co(NO_3)_2 \times 6H_2O$ – 0.044.

Culturing conditions: Illumination cycle consisted of a light phase (8 h) and dark phase (16 h), periodic stirring (2–3 times per day), the effect of temperature was considered (28 ± 1°C), conical flasks with cotton-gauze stopper, and sterile conditions. An aquarium lamp (Tetronic LED) that provides red (4350 ± 50 lx) and blue (2450 ± 50 lx) light, and a lamp (LLED-01 LED light) that provides white light (2150 ± 50 lx) were used to study the effect of illumination color on EPS production by microalgae. White light is a mixture of light of different wavelengths from the violet visible light range (0.42 µm) [26]. For blue light, the reference wavelength of the light filter λ was 874 nm and formed a reflection band with a midpoint at wavelength λ0 = 437.5 nm. The refractive indices of ZnS and MgF_2 at the reference wavelength took values of 2.3036 and 1.3861, respectively. The bandwidth in such case was in the interval from λ_1 = 307 nm to λ_2 = 735 nm [27]. The red light filter allowed only rays of red light to pass through and absorbed the rest. The intensity of light hitting the microalgae samples was 13 klx [28]. The lamps were placed at a distance of 15 cm from the flasks with microalgae cultures.

The lamps were placed at a distance of 15 cm from the flasks with microalgae cultures. The concentration of exopolysaccharides in the culture fluid was determined by the phenol-sulfuric acid method at OD480. The relative specific EPS concentration (g/g) was calculated as the ratio of the EPS concentration of the culture fluid (g/L) to the cell concentration in the suspension (g/L).

Optimization of the chemical composition of the nutrient medium was carried out using mathematical modeling: A full factorial two-level experiment (FFE). The functions are cellular concentration and specific concentration of EPS. The macrosols included in the nutrient medium were taken as the studied factors: Nitrogen source – KNO_3; phosphorus source – KH_2PO_4; magnesium source – $MgSO_4 \times 7H_2O$, and chlorine source – NaCl [Table 1].

Microalgae samples (*Porphyridium* sp. Nag. P-519, *P. cruentum* P-271, and *P. sordidum* P-293) were cultivated under sterile conditions in conical and round bottom flasks with cotton-gauze plugs, using nutrient media selected at the previous stage of the study under the following technological parameters: Working temperature of cultivation for microalgae strains *Porphyridium* sp. Nag. P-519 and *P. cruentum* P-271 - 25°C; *P. sordidum* P-293; - 45°C; light/darkness for *Porphyridium* sp. Nag. P-519 - 16/8 h, for *P. sordidum* P-293 - 12/12 h, for *P. cruentum* P-271 - 8/16 h, white light with an intensity of 5000 lx, duration – 21 days [25,26]. The studied microalgae samples were subjected to constant stirring in a thermostated shaker with a rotor speed of 100 rpm during the experiment.

Table 1: Planning matrix of a full factorial experiment.

Component	Factor	Concentration of components, g/L			
		Lower level «–»	Upper level «+»	Control	Step
KNO_3	X_1	0.620	1.860	1.24	0.620
$K_2HPO_4 \times 3H_2O$	X_2	0.325	0.975	0.65	0.325
$MgSO_4 \times 7H_2O$	X_3	1.250	3.750	2.50	1.250
NaCl	X_4	6.260	18.780	12.52	6.260
Matrix plan No.		X_1	X_2	X_3	X_4
1		–	–	–	–
2		+	–	–	–
3		–	+	–	–
4		+	+	–	–
5		–	–	+	–
6		+	–	+	–
7		–	+	+	–
8		+	+	+	–
9		–	–	–	+
10		+	–	–	+
11		–	+	–	+
12		+	+	–	+
13		–	–	+	+
14		+	–	+	+
15		–	+	+	+
16		+	+	+	+
17		Control			

To isolate endopolysaccharides, the suspended sediment of microalgae culture fluid was dissolved in distilled water and ultrasonic dispersion was performed in an Ultrasonic Processor (Antylia Scientific, USA) with varying power (20 W, 40 W, and 60 W) and treatment duration (2.0 min) [29]. The mass of endopolysaccharides was then measured by the anthrone-sulfuric acid method [21] and converted to dry biomass (mg/g d.m.). For this purpose, 150 µL of anthrone agent (0.1% solution of recrystallized anthrone in concentrated sulfuric acid) was added to each well of a microplate (DV-expert, Moscow, Russia) containing 50 µL of samples. The plates were then placed in a Pozis RK-102 S refrigerator (Diamond Elektrik, Moscow, Russia) for 10 min at 4°C. After cooling, the samples were incubated in an A-24 thermostat (Millab, Moscow, Russia) for 20 min at 70°C. After heating, the samples were cooled to room temperature. The optical density was measured at 620 nm. The standard curve was plotted using sucrose solutions [30].

Polysaccharide solutions were purified from ballast substances by electrodialysis, which allows simultaneous dialysis and concentration of aqueous polysaccharide solutions [31]. The resulting polysaccharide solution was dialyzed with deionized water using a dialysis bag with an MWCO of 3500 Da, resulting in a non-dialyzable sample inside the dialysis bag. A 3-fold volume of absolute ethanol was added to the non-dialyzable sample solution for precipitation at 4°C for 8 h. The precipitate was collected and lyophilized under vacuum using Free-Zone 12 lyophilizer (Labconco, USA) to obtain the target Ps.

Statistical analysis was performed using SPSS and Excel 2016 program. The chemical composition of the nutrient medium was optimized by adjusting the amounts of the nitrogen source (KNO_3), phosphorus source (X_2PO_4), magnesium source ($MgSO_4 \times 7H_2O$), and chlorine source (NaCl) added to promote the accumulation of biomass and Ps. Analysis of variance was performed using the Kraskell–Wallis criterion (chi-square; $P < 0.05$), Mann–Whitney criterion with Bonferroni correction for pairwise comparison (z; $P < 0.016$), and Pearson's coefficient to test for the presence of correlation (r; $P < 0.05$). All experiments were repeated 5 times.

3. RESULTS AND DISCUSSION

The metabolism of microorganisms is unusually sensitive to environmental conditions. Microorganisms are in close contact with the nutrient medium which they inhabit and respond to changes in the set and ratio of its composition. A change in the concentration of any component within the limits that allow microorganism development can significantly affect cell metabolism [Table 1].

Methods of mathematical planning of the experiment are used to identify such a composition of the nutrient medium at which the desired effect (biomass yield and accumulation of certain metabolic products) is observed. Mathematical planning enables the simultaneous study of several factors and quantification of their influence on the production of the target substance [32].

KNO_3, $K_2HPO_4 \times 3H_2O$, $MgSO_4 \times 7H_2O$, and NaCl considered in this work served as a source of nitrogen, phosphorus, magnesium, and sulfur for microalgae, respectively [33]. A fourth factor, sodium chloride concentration, was chosen to optimize the salinity of the nutrient medium [34].

The regression equations were obtained as a result of FFE. These equations are shown in Table 2 and have been formulated while considering the statistical significance of factors and checking the mathematical model for adequacy.

For each of the three strains studied, an inversely proportional relationship between KNO_3 concentration in the nutrient medium and EPS production was found, that is, the lower the KNO_3 concentration, the more EPS produced by the microalgae. It was also found that EPS production by strains P-271 and P-293 was statistically significantly affected by the presence of sodium chloride. For P-271, the relationship is inversely proportional, while for P-293, the relationship is directly proportional.

The FFE results are graphically summarized in Figure 2. The leader in biomass growth is P-271 (Matrix Plan No. 10, 13–15) with a maximum of 0.8 g/L. For strain P-293, variants No. 2, 11, and 14 (0.6 g/L) were the best. With respect to P-519, the initial nutrient medium composition (control) was the best for biomass growth, 0.7 g/L on average.

When evaluating the specific EPS concentration, it was revealed that among the presented strains, strain P-293 is the undisputed leader (2.25 g/g on average) under conditions of nitrogen source deficiency and sodium chloride excess (No. 9 and 13 of the matrix plan). The maximum EPS yield (1.6 g/g) for strain P-519 was observed at No. 15 of the matrix plan. Strain P-271 was distinguished by the lowest value of EPS, averaging 1 g/g (No. 8 of the matrix plan).

Investigation of the effect of the light [Figure 3] on EPS production by red microalgae revealed that the highest values were observed on the 5th day of cultivation under red-white light conditions for P-293 and P-519 compared to white and blue light. For strain P-271, more EPS is produced under white-light conditions compared to red-white or blue-light conditions.

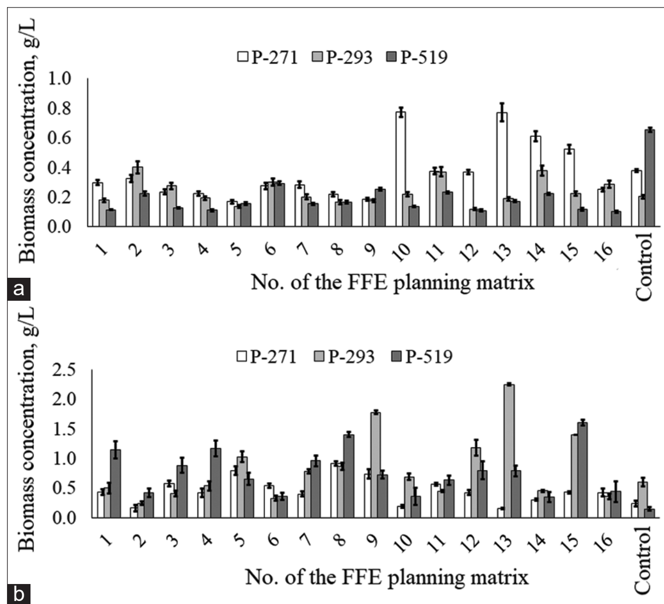

Figure 2: Effect of nutrient medium composition on biomass concentration (a) and specific EPS concentration (b).

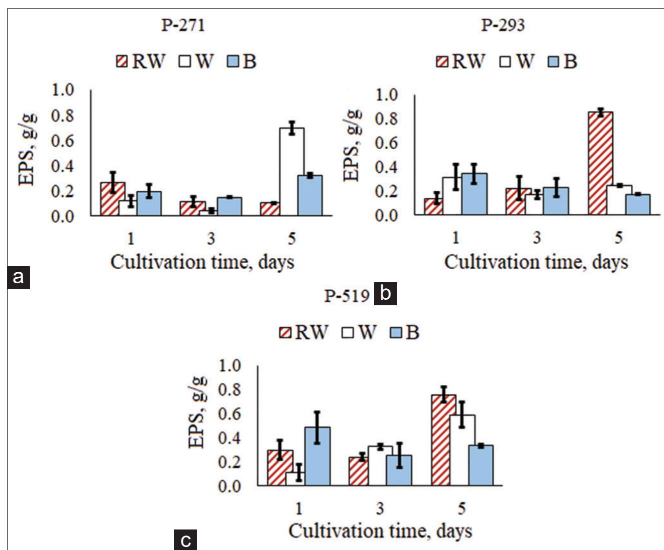

Figure 3: Comparison of specific EPS concentration when cultivated under different light conditions: RW: Red-white light; W: White light; B: Blue light: P- 271 (a), P-293 (в), P-519 (c).

Analysis of variance [Table 3] using the Kraskell–Wallis criterion (Chi-square; *P*) showed the presence of intergroup differences (RW/W/B) on the 5th day of cultivation in the conditions with respect to both P-271 (6.50; 0.039), P-293 (12.50; 0.002), and P-519 (12.04; 0.002).

Under red-white illumination, the concentration of extracellular polymeric substances (EPS) increases in P-293 and P-519 cultures during cultivation. Under white light conditions, an EPS increase was observed for strains P-519 and P-271. The use of blue lamp was found to decrease EPS in the culture fluid of P-293 and P-519. Under white-light conditions, the level of EPS decreased in P-293, and under red-white light conditions in P-271 [Table 4].

The chemical composition of the exopolysaccharides of *Porphyridium* strain P-271 is represented by D-xylose, D-glucose, D- and L-galactose,

Table 2: Results of a full factorial experiment.

Strain of *Porphyridium* sp.	Regression equation
Porphyridium cruentum P-271	$y=0.515–0.065×X_1–0.060×X_4$
Porphyridium sordidum P-293	$y=0.086–0.196×X_1+0.159×X_4$
Porphyridium sp. Nag P-519	$y=1,127–0,236×X_1$

Table 3: Results of intergroup comparison (day 5).

Lighting conditions	P-271	P-293	P-519
RW/W	−2.402; 0.016*	−2.611; 0.009*	−1.567; 0.117
RW/B	−2.619; 0.009*	−2.611; 0.009*	−1.567; 0.117
W/B	−2.619; 0.009*	−2.611; 0.009*	−2.611; 0.009*

The z-value of the criterion and the p–value are given; * - statistical significance of the intergroup difference, RW: illumination with red-white light; W: illumination with white light; B: illumination with blue light

Table 4: Results of regression analysis.

Strain	RW		W		B	
	r	*P*	r	*P*	r	*P*
P-271	0.0003	−0.795**	0.0750	0.472	0.0630	0.492
P-293	0.0000	0.888**	0.3060	−0.283	0.0003	−0.795**
P-519	0.0100	0.643**	0.0000	0.945**	0.2800	−0.567*

r is the value of the Pearson correlation coefficient, */** is the statistical significance of r, RW is red–white light illumination; W is white light illumination; B is blue light illumination

3-O-methylxylose, 3- and 4-O-methylgalactose, and D-glucuronic acid in approximate molar ratios of 3:1:2.5:0.13:0.13:0.13:0.8. *Porphyridium* P-293 contains 2-O-methylhexose and 2-O-methylglucuronic acid, whereas the exopolysaccharides of *Porphyridium* strain P-519 lack these two sugars but contain 2,4-di-O-methylgalactose. Xylose, glucose, and galactose are present in the exopolysaccharides of all three microalgae strains as end groups and 1,3- and 1,4-linked residues, with galactose and glucose also present as 1,3,4-linked or sulfated residues [35].

It is known that an increase in the carbon/nitrogen (C/N) ratio can promote the production of EPS by *P. purpureum* [21,25]. As the result of this research, factor X_1 was used for KNO_3, the lower limit was 0.620, the upper limit was 1.860, the control was 1.24, and the step between levels was 0.620. For $K_2HPO_4x3H_2O$, factor X_2 was used, the lower limit was 0.325, the upper limit was 0.975, and the control was 0.650 with a step of 0.325. For $MgSO_4$, factor X_3 was used, the lower limit was 1.250, the upper limit was 3.750, and the control was 2.50 with a step of 1.250. For NaCl, factor X_4 was used, the lower limit was 6.260, the upper limit was 18.750, and the control was 12.520 with a step of 6.260.

The regression equation for *P. cruentum* P-271 is as follows $y = 0.515–0.065 × X_1–0.060 × X_4$; for *P. sordidum* P-293, $y = 0.086–0.196 × X_1 + 0.159 × X_4$; for *Porphyridium* sp. Nag P-519, $y = 1.127–0.236 × X_1$. For *P. sordidum* strain P-293, the maximum EPS yield of 1.83 g EPS/g microalgae was observed when the concentration of NaCl in the nutrient medium was increased and the concentration of KNO_3 was decreased. For the strain *Porphyridium*sp. Nag. P-519, the maximum EPS yield (almost 0.5 g/L) was observed when the concentration of NaCl in the nutrient medium was increased. The highest EPS yield for *P. cruentum* strain P-271 was obtained when $K_2HPO_4·3H_2O$ and NaCl were used, amounting to 0.4 EPS/g of microalgae. Optimization of technological conditions of microalgae cultivation contributes to an

increase in cell growth rate, biomass production, and polysaccharide yield [36]. Temperature can affect polysaccharide production in combination with light energy, as it affects nutrient uptake and cell wall structure [37]. The optimum temperature required for culturing microalgae ranges from 15 to 30°C; beyond this temperature range, damage or death of microalgae cells may occur [38].

Blue and red light are known to favor the growth and EPS production of microalgae [39]. Contrarily, blue light (430 nm) was found to be the least beneficial to *P. sordidum* growth and EPS production in these studies. The light spectrum was also recognized to critically affect the EPS composition of microalgae, as the results showed that both blue (400–500 nm) and red (600–700 nm) light effectively increased the polysaccharide production of *P. cruentum* [40]. The study of light as the main parameter to control the growth and polysaccharide production of *P. cruentum* showed that blue light with wavelengths between 400 and 500 nm can be used to stimulate cell growth and increase polysaccharide production up to 4.63 g/L [41]. There are reports on the effects of light with different wavelengths on polysaccharide production. Blue light was reported to be an effective tool to improve *P. cruentum* cell growth and polysaccharide synthesis [42]. Our findings are consistent with those of other studies [9-35]. The production of exopolysaccharides and other valuable biologically active substances by *Porphyridium* microalgae (P-271, P-293, and P-519) was enhanced when exposed to white, blue, and red light [28].

In comparison to other microalgae exopolysaccharides, the profile of *Porphyridium* (P-271, P-293, and P-519) makes it an alternative source of exopolysaccharides with a high purity index classified as food grade [43]. Exopolysaccharides are also used as active compounds in the medical and cosmetic industries because they have antioxidant [44], antimicrobial, and anticancer properties.

4. CONCLUSION

The obtained results can be succinctly summarized. EPS production by strain P-271 is equally affected by both the chemical composition of the medium (nitrogen and sodium chloride deficiency) and the type of light (white light). The chemical composition of the medium (such as nitrogen and sodium chloride deficiency) had a greater effect on EPS production by strain P-293 compared to the type of light (red-white light), which had a smaller effect. EPS production by strain P-519 is equally affected by the chemical composition of the medium (nitrogen deficiency) and the type of light (white or red-white light). More research in this area is planned to assess the mutual influence of light factors and medium chemical composition on polysaccharide production by red microalgae. The findings of this study will serve as the foundation for a technology for cultivating *Porphyridium* microalgae (P-271, P-293, and P-519) and producing exopolysaccharides that are cost-effective, simple to use, and reproducible in any desired volume. It will enable larger-scale production of high-value-added exopolysaccharides [29].

Microalgae Ps are known to have anti-inflammatory, antibacterial, antiviral, immunomodulatory, and anticarcinogenic activities. More research on polysaccharide production and properties is planned to evaluate microalgae preparations and supplements for lowering blood cholesterol levels and preventing the formation of cholesterol plaques. It is possible to establish intestinal blood flow, populate the intestines with beneficial bacteria, accelerate the elimination of food and chemical mutagens from the body, and much more with the help of microalgae Ps. It will be possible to create products for weight loss, anti-age, sports nutrition, and maintaining a healthy lifestyle using microalgae exopolysaccharides. The plan is to look into the potential of preparations based on microalgae exopolysaccharides to prevent diabetes, hypertension, anemia, peptic ulcers, and other diseases. All of these are future research directions for the Ps obtained in our study.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

6. FUNDING

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7. CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

10. PUBLISHER'S NOTE

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