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Effect of Different Concentrations of NaCl on Growth and Biochemical Characteristics of Red Microalga *Porphyridium cruentum*

Juliana Ivanova[1], Tanya Toshkova-Yotova[1], Lyudmila Kabaivanova[2*]

[1]*Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Bulgaria*
[2]*The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Bulgaria*

Abstract

The red microalga *Porphyridium cruentum* is a potential source of valuable biologically active compounds. This microalga is easily cultivated and could change its growth and biochemical composition in response to environmental variations. The effect of growth medium content, especially salinity (15, 20, and 27 gL^{-1} NaCl) on *P. cruentum* cell growth, biomass composition, and extracellular polysaccharides production was investigated in this study. The results showed that the highest biomass yield and phycoerythrin content was achieved when NaCl in a concentration of 20 gL^{-1} was introduced into the culture medium. The measured biomass was equal to 4.3±0.1 gL^{-1} compared to 3.3±0.1 gL^{-1} for the control sample at the end of the cultivation process. The amount of chlorophyll „a“ was also 1.33 times greater in the sample with 20 gL^{-1} NaCl compared to the control sample with 27 gL^{-1}, where the carotenoid content was higher. The highest amount of extracellular polysaccharide was accumulated by the microalga at 27 gL^{-1} NaCl. The measured viscosity was 3.35 mPa.sec - 1.4 times more than the sample with 20 gL^{-1} NaCl. The investigations conveyed lead to the conclusion that with only slight changes in the salt concentration, a targeted biosynthesis could be achieved, nevertheless, all other conditions remained the same. The study provides an opportunity to optimize growth and metabolite production using optimization in the cultivation process of the microalgae.
Keywords: *Porphyridium cruentum*, NaCl, growth, metabolites production

Резюме

Червеното микроводорасло *Porphyridium cruentum* е потенциален източник на ценни биологично активни съединения. Това микроводорасло се култивира лесно и може да промени своя растеж и биохимичен състав в отговор на промените в околната среда. Ефектът от съдържанието на растежната среда, особено солеността (15, 20 и 27 gL^{-1} NaCl) върху клетъчния растеж на *P. cruentum*, състава на биомасата и производството на извънклетъчни полизахариди беше изследван в това проучване. Резултатите показват, че най-високият добив на биомаса и съдържанието на фикоеритрин се постигат, когато NaCl в концентрация от 20 gL^{-1} се въведе в културалната среда. Измерената биомаса е равна на 4.3±0.1 gL^{-1} в сравнение с 3.3±0.1 gL^{-1} за контролната проба в края на процеса на култивиране. Количеството на хлорофил „a“ също е 1,33 пъти по-голямо в пробата с 20 gL-1 NaCl в сравнение с контролната проба с 27 gL^{-1}, където съдържанието на каротеноиди е по-високо. Най-голямото количество извънклетъчен полизахарид се натрупва от микроводораслото при 27 gL^{-1} NaCl. Измереният вискозитет е 3.35 mPa/sec - 1,4 пъти повече от пробата с 20 gL^{-1} NaCl. Изследванията водят до заключението, че само с леки промени в концентрацията на сол може да се постигне целенасочена биосинтеза, въпреки че всички останали условия остават едни и същи. Проучването дава възможност за оптимизиране на растежа и производството на метаболити чрез оптимизиране в процеса на култивиране на микроводораслите.

Introduction

A successful biotechnological process depends on the selection of a suitable organism capable of producing a desired product at appropriate conditions (Ivanova *et al*., 2015; Daneshvar *et al.*, 2021). Microalgae are primary producers found in the oceans. They use light energy and carbon dioxide to convert them into biomass rich in mainly carbohydrates, proteins, and lipids as well as many

* Corresponding author: lkabaivanova@yahoo.com

other important biologically active molecules (Harwood *et al*., 2009). As a promising source of biomass for use in practice, microalgae have the most outstanding advantages of high growth rate and high photosynthetic rate in specific environments. They are 10 times more capable of fixing CO_2 than higher plants (Ho *et al.,* 2013).

Porphyridium cruentum (Rhodophyta) is a single-celled red microalga with high salt tolerance, that can synthesize valuable bioactive substances such as phycoerythrins, extracellular polysaccharides, and polyunsaturated fatty acids during their growth process (Hao-Chan *et al.,* 2022). Microalgae are capable of living freely or colonizing marine waters and can be cultivated in a fast life cycle. The unique bioactive substances synthesized find different applications - in medicine, pharmacy, cosmetics, the food industry, animal feeding, agriculture, etc. (Lu *et al.,* 2020). *P. cruentum* cells are used to produce phycobiliproteins (phycoerythrin, phycocyanin, and alo-phycocyanin). The main application potential of these molecules is as a natural dye, but several scientific studies have demonstrated numerous biological properties and a wide range of pharmaceutical applications (Li *et al*., 2019). Phycoerythrin has the advantages of high fluorescence intensity, anti-oxidation, and scavenging free radicals. Thus, it exhibits broad commercial value in food and medicine (Qiu *et al.*, 2004; Bueno *et al*., 2020).

The unsaturated fatty acids omega-3 (ω-3) contained in algal cells such us α-linolenic acid (C18H30O2, ALA), eicosapentaenoic acid (C20H30O2, EPA), and docosahexaenoic acid (C22H32O2, DHA) can lower blood cholesterol levels and thus reduce the risk of cardiovascular diseases and protect arteries (Brenna *et al.*, 2009).

The cells of these algae are encapsulated within a cell wall of a polysaccharide complex, the external part of which ("soluble polysaccharide") dissolves in the medium, thus increasing its viscosity (Arad *et al*., 2010). Extracellular polysaccharides of red microalgae exhibit a huge amount of bioactivities that have nutritional (Vasileva *et al.,* 2019), and medical significance (Risjani *et al*., 2021; Ivanova *et al*., 2022).

According to previous studies, microalgae can respond to changing environmental conditions by regulating their synthesis of metabolites, so that changes can be used to achieve an accumulation of required substances (Paliwal *et al.*, 2017). The suitable culture conditions for specific microalgae species are vitally important for their optimal growth and biochemical production. The main environmental factors affecting the growth of microalgae are nitrogen and phosphorus concentrations, salinity, temperature, light, and pH (Haris *et al.,* 2022). Therefore, these culture parameters should be optimized to improve biomass productivity. Salinity is an important factor affecting microalgal growth (Xia *et al*., 2014).

The aim of the present study was to investigate and follow the influence of salinity in the culture medium on microalgal growth and biochemical characteristics for better biotechnological applications of *P. cruentum*.

Materials and Methods

Strain and growth conditions

Monoalgal, non-axenic cultures of *P. cruentum* (AG.) NAG Vischer 1935/107 (Rhodophyta, Porphyridiales, Porphyridiaceae) from the culture collection of the Institute of Botany ASCR, Třeboň, Czech Republic) were grown autotrophically in 200 mL flasks, at 22°C under continuous illumination (white fluorescent light, 132 µmol photons $m^{-2}s^{-1}$) for 7 days (168h). The cultures were continuously supplied with 2% CO_2. The initial culture density was 0.8 gL^{-1}. Algal cultivation was carried out using a nutrient medium (Hemerick, 1973) with three different concentrations of NaCl: 27 gL^{-1} (the standard nutrient medium), 15 g L^{-1}, and 20 g L^{-1}.

Analytical methods

After harvesting the samples, algal suspensions (3×5 mL each) were filtered through Whatman GF/C glass filters (WHATMAN INTERNATIONAL LTD, Maidstone, UK) and oven-dried at 105°C to a constant weight. Algal growth was evaluated gravimetrically by measuring the increase in biomass dry weight (DW).

Pigments

Chlorophyll "a", chlorophyll "b" and carotenoids were measured after extraction with boiling methanol and the quantity was calculated using Mackinney formulas (Mackinney, 1941). Phycobiliproteins were extracted with 0.01 M potassium phosphate buffer (pH=6.7) from homogenized cells (vibrations homogenisator VHG1, Germany, 4°C, 10 min). The quantities were calculated according to the equations of Siegelman and Kycia (Kycia, 1978).

Analytical methods

Total protein content was measured according to the method of Lowry (Lowry, 1951), with BSA as a standard. Total carbohydrates were quantified

as glucose equivalents by the phenolsulfuric acid method (DuBois *et al*., 1956). The lipid extraction was conducted by the method of Petkov and Dilov, (1987). Centrifuged algal cells were used for the extraction with hot ethanol under reflux and then re-extracted with chloroform. The lipid extracts were released from the chloroform at 40–45°C on a rotary vacuum evaporator and the lipid quantity (g L^{-1}) was determined gravimetrically. All spectrophotometrical analyses were performed using a T70 UV/Vis (PG INSTRUMENTS LTD, Leicester, UK).

Extracellular polysaccharide

The viscosity of the culture supernatant shows the amount of extracellular polysaccharide synthesized. It was measured by a viscosimeter B3 (VEB MLW, DDR). Samples for all biochemical analyses and the viscosity were obtained during the last day of the cultivation for each of the cultures. The pH of the cultures was measured daily via a pH meter (INOLAB pH 7110, Ankara, Turkey).

Statistical analysis

All experiments were conducted in three independent biological replicates and each measurement had three replicates. The data were presented as the means ± standard deviation. The significance of differences between the treatments was evaluated by ONE WAY analysis of variance (ANOVA) and Bonferroni's post hoc test using GRAPHPAD INSTAT SOFTWARE (San Diego, CA, USA). *Light microscopy* visualization of algal cells was carried out by using an Olympus BX50 microscopeValues of $P < 0.05$ were considered significant.

Results and Discussion

The red microalga *P. cruentum* was cultivated for 7 days at three different salt concentrations: 15, 20, and 27 gL^{-1} NaCl (Fig. 1). Cell growth evolution was followed through dry weight (Fig. 2).

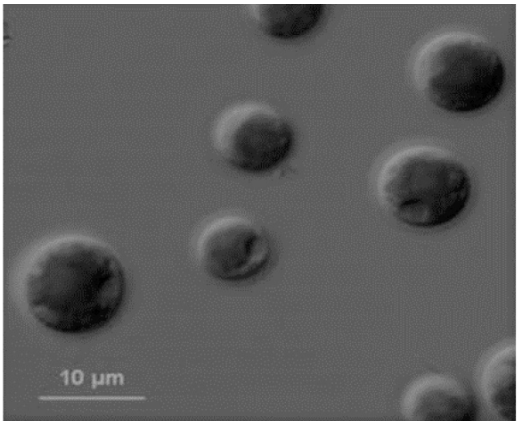

Fig. 1. Visualization of *P. cruentum* growth in 20 gL^{-1} NaCl-supplemented medium

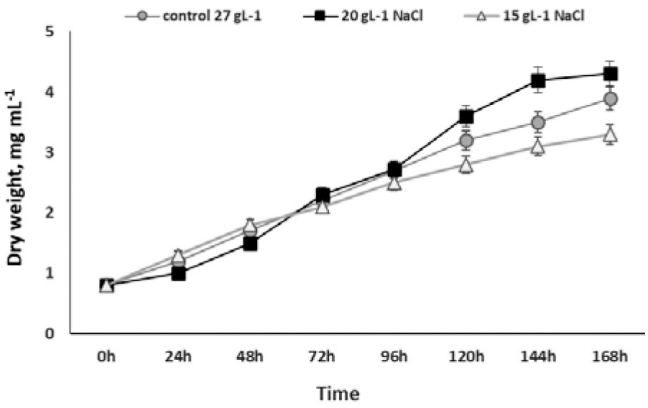

Fig. 2. *P. cruentum* growth pattern under different sodium salt concentrations -15, 20, and 27 gL^{-1} NaCl

The sample with 27 gL^{-1} NaCl is taken as a control sample because the medium for cultivating the algae has this defined salinity concentration. Maximum growth by estimated DW content of 4.3 g/L was achieved for salinity of 20 gL^{-1} at the 168th h of cultivation. For the sample with 27 gL^{-1} NaCl the estimated DW was 3.9 gL-1, which was 1.18 times higher than the DW achieved for the salinity of 15 gL^{-1} (DW of 3.3 gL^{-1}).

Ferreira *et al.* (2021) studied the effect of growth medium salinity (18, 32, and 50 gL^{-1} NaCl) on the growth of *P. purpureum* **s**train isolated from a lagoon in Portugal. Cultural conditions were pH of 9.2, air bubbling (without CO_2 addition), and natural sunlight to maintain a photoperiod of 12:12 h light:dark. After 19 days of growth, the results showed that the optimal salt concentration for the growth of these microalgae was 32 gL^{-1} salinity. These results do not match ours, but the studied cultivation conditions and strain were different. Five locally isolated microalgae - *Amphidinium carterae*, *Nephroselmis* sp., *Tetraselmis* sp., *Asteromonas gracilis,* and *Dunaliella sp.* were examined by Hotos *et al*. (2021) as laboratory batch cultures. The five species examined exhibited different responses in the salinities used, whereby *Amphidinium* clearly performed best in 20 ppt. *Nephroselmis* and *Tetraselmis* grew almost in the same way in 20 and 40 ppt. *Asteromonas* performed best in 100 ppt, although it grew quite well in both 40 and 60 ppt. *Dunaliella* grew equally well in all salinities (20, 40, 60 ppt). The results of this study showed that the optimal concentration of NaCl depends on the type of investigated strain. Knowing the optimal salinity could favor good growth.

The pigment content of algae is an important factor for the viability of cells. It is again a specific characteristic for each species. Pigment evaluation

is an indirect measure of cell growth (Masojídek *et al*., 2013). On the other hand, pigments can serve as a valuable bioproduct used in various applications: medicine, cosmetics, and the food industry (Li *et al*., 2019). A comparison of the results for the total amount of pigments of *P. cruentum* at the 168th hour of cultivation for the three studied salinities was carried out. The results showed an increase in the entire amount of pigments by about 1.04 times for the sample with 20 gL^{-1} NaCl compared to the control sample (Table 1).

Table 1. Sum of pigments, % of DW

	Control - 27 gL^{-1} NaCl	20 gL^{-1} NaCL	15 gL^{-1} NaCl
Sum of pigments, % of DW	6.1	6.35	4.78

As shown in Figure 3, at the 168th h of cultivation the quantity of chlorophyll "a" was the biggest at salinity of 20 gL^{-1} (1.65% of DW), followed by that at salinity 27 gL^{-1} and 15 gL^{-1}.

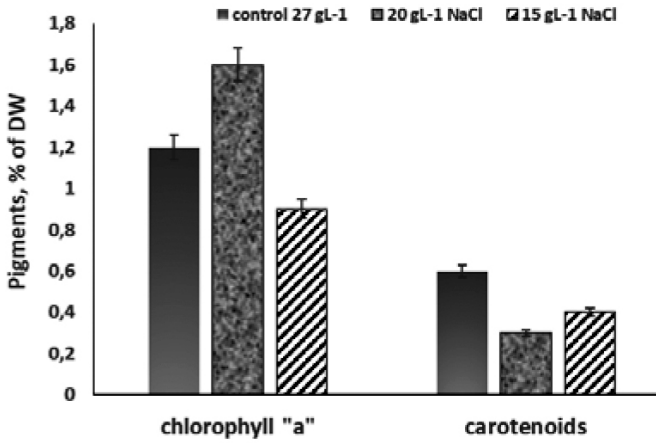

Fig. 3. Pigment content of *P. cruentum* at different NaCl concentrations

Analysis of the water-soluble phycobiliproteins showed an increased amount of phycoerythrin in the 20 gL^{-1} NaCl sample about 1.10 times compared to the control sample (Fig. 4). It is well known that phycoerythrin is the most important pigment in the cells of these red algae, as it is increasingly used in practice. Phycobiliproteins have many potential applications in foods, cosmetics, medical diagnosis, and treatment of diseases (Li *et al*., 2019).

Algae-produced exopolysaccharides serve as a molecular glue that allows cells to adhere to each other and helps build a stronger biofilm. (Limoli *et al*., 2015) On the other hand, the valuable properties of extracellular polysaccharides are increasingly used in biomedicine (Ivanova et al., 2022; Zheng *et al*., 2022). Therefore, it is relevant to study and

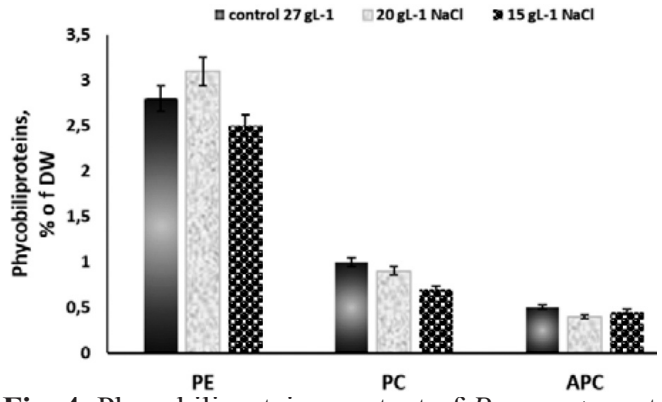

Fig. 4. Phycobiliproteins content of *P. cruentum* at different NaCl concentrations; PE- phycoerythrin; PC - phycocyanin; APC – allophycocyanin.

develop new strategies to increase the production of exopolysaccharides with the aim of biotechnological and medicinal applications. We recorded the highest viscosity of 3.35 mPa.sec - about 1.67 times more compared to the sample with the lowest salinity in the control medium with 27 gL^{-1} NaCl (Fig. 5). Although the growth of the alga is slightly better at a salt concentration of 20 gL^{-1} if the strain is used for biotechnological production of extracellular polysaccharides, it would be appropriate to increase the amount of NaCl to the optimal for its production, level (Raposo *et al*., 2013).

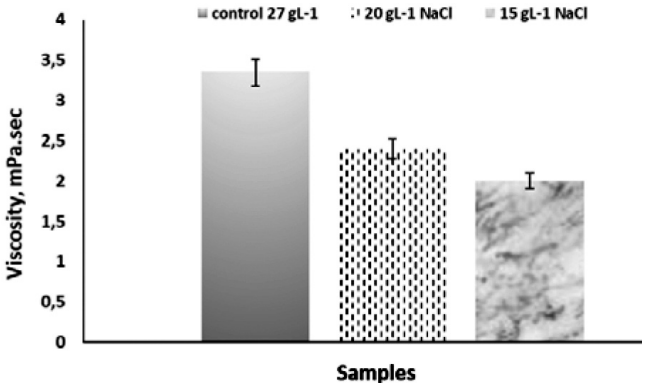

Fig. 5. Viscosity of extracellular polysaccharide at different NaCl concentrations at the end of cultivation.

A significant difference in the results for the percentage of proteins and carbohydrates in microalgal cells at different concentrations of NaCl was not registered. However, there exists some predominance of carbohydrate accumulation in the control sample (Table 2).

Lee *et al*. (1988) reported that the total lipids content of the *P. cruentum* culture was more at lower salinities (12 gL^{-1}) in the stationary phase. Similar results for the percent content of lipids were obtained in this study. We found the highest percentage of lipids from the dry weight in the sample

Table 2. Biochemical characteristics of *P. cruentum* at different NaCl concentrations.

Metabolites, % of DW	27 gL^{-1} NaCl (Control)	20 gL^{-1} NaCL	15 gL^{-1} NaCl
Proteins	29	31	33
Carbohydrates	59	56	49
Lipids	8	9	12
Sum	95	95	94

with 15 gL^{-1} NaCl, about 1.5 times more than the control sample.

Conclusion

The effect of salinity/NaCl on *P. cruentum* cell growth, biomass composition, pigments content, and extracellular polysaccharides production was revealed. Highest biomass yield, phycoerythrin and chlorophyll „a“ synthesis was achieved when NaCl in a concentration of 20 gL^{-1} was introduced into the culture medium, while the highest amount of extracellular polysaccharide was accumulated by the microalga at 27 gL^{-1} NaCl under the described cultivation conditions. The investigations conveyed lead to the conclusion that with only slight changes in the salt concentration, a targeted biosynthesis could be achieved, nevertheless, all other conditions remained the same. The study provides an opportunity to increase growth and metabolite production using optimization in the cultivation process of the red microalga by salinity alterations.

Acknowledgment

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