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BIOTECHNOLOGICAL PERSPECTIVES OF THE RED MICROALGA *PORPHYRIDIUM CRUENTUM*

Ivanina Vasileva[1], Svetoslav Alexandrov[1], Juliana Ivanova[1]*
[1]Experimental Algology Department, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences

Abstract: The red microalga *Porphyridium cruentum*, a potential source of bioactive substances, was the subject of a study on the effect of nutrients and different CO_2 concentrations (0.04 and 2%) on the growth, biochemical composition and synthesis of phycobiliproteins and polysaccharides. For this purpose the alga was grown on two nutrition media, referred in this manuscript as M1 and M2 (M1 is richer in chloride and nitrogen substances). We have estimated that the abovementioned parameters varied under different cultivation conditions. Best yield was observed in M1 in the presence of 2% CO_2, with a peak of the measured biomass equal to 4.0±0.1 g L^{-1}. On the other side, compared to M1, M2 stimulated the pigment synthesis when the culture was enriched with 2% CO_2. The highest amount of carbohydrates and pigments was observed when the alga was grown with 0.04% CO_2. These results confirm that *Porphyridium cruentum* posesses great adaptive capabilities that can be used in future biotechnological practices.

Keywords: growth, biochemical composition, polysaccharide, phycobiliprotein, CO_2 concentration

INTRODUCTION

Microalgae are a vast group of aerobic photosynthetic microorganisms that thrive in various habitats (Sharma, 2007; Phang, 2008). They are suitable producers of biomass which is a valuable raw material for different industries (pharmaceutical, cosmetic, food, etc.), because of the compounds that can be extracted from it. Such commercially applicable compounds include pigments (β-caroten, astaxanthin, phycocyanin, phycoeritryn), polysaccharides, unsaturated fatty acids and proteins. *Porphyridium cruentum* is a subject of great interest due to its high levels of productivity of sulphated polysaccharides (Gouveia et al., 2008), which have been proved to possess different biological activities – antioxidant (Tannin-Spitz et al., 2005), anti-inflammatory (Matsui et al., 2003), anti-tumor (Gardeva et al., 2009), antiviral (Minkova et al., 1996; Raposo et al., 2014), immune stimulating (Sun et al., 2012) and many others. Phycobiliproteins are deep-colored, water-soluble proteins that are present mainly in Cyanobacteria and Rhodophyta. They capture light energy, which is then passed on to the chlorophylls during photosynthesis. Also, they can neutralize the reactive oxygen species due to their chemical structures and chelating properties, thus reducing oxidative stress (Christaki et al., 2015).

All of this is a premise for a further development of the algal biotechnology by studying the physiology and the biochemistry of the microalgae. In this study, we tried to determine how the changes in cultivation conditions of *Porphyridium cruentum* affect the composition of the biomass and the amount of the extracellular polysaccharides. The purpose was not only to acquire a higher amount of biomass, but to achieve better production of polysaccharides and/or pigments and at the same time to maintain stress-free conditions.

MATERIALS AND METHODS

Strain and growth conditions

Monoalgal, non-axenic cultures of *Porphyridium 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 either with 0.04% (atmospheric air) or 2% CO_2. The initial culture density was 0.5 g L^{-1}. The content of the used nutritive media were shown on Table 1.

Tab. 1.
Content of the modified nutritional media for the growth of the red *Porphyridium cruentum* (based on the medium of Brody & Emerson, 1959).

	Elements	Medium 1 [g L^{-1}]	Medium 2 [g L^{-1}]
1	K_2HPO_4	0.8	0.66
2	KNO_3	1	-
3	KCl	-	16
4	NaCl	27	12.5

Correspondence: Juliana Ivanova, Experimental Algology Department, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences. Acad. G. Bonchev Str., bldg. 21, 1113, Sofia, Bulgaria. Tel.: +359 2 9792118; Fax: +359 2 8739952; e-mail: juivanova@yahoo.com.

5	KI	-	0.05
6	KBr	-	0.05
7	$MgSO_4.7H_2O$	6.6	2.5
8	$MgCl_2.6H_2O$	5.6	-
9	$CaCl_2.2H_2O$	1.5	-
10	$Ca(NO_3)_2.4H_2O$	-	0.25
11	$NaHCO_3$	$4x10^{-5}$	-
trace elements [mg L^{-1}]			
1	H_3BO_3	2.86	2.86
2	$MnCl_2.4H_2O$	1.83	1.83
3	$CuSO_4.5H_2O$	1	1
4	$ZnSO_4.7H_2O$	0.2	0.2
5	MoO_3	0.2	0.2
6	NH_4VO_3	0.25	0.25
7	$Na_2WO_4.2H_2O$	0.2	0.2
8	TiO_2	0.013	0.013

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).

Specific growth rate (μ) was calculated as follows: $\mu = \ln(mt_2/mt_1)/t_2–t_1$ (Levasseur et al., 1993), where mt_1 and mt_2 represent the cells DW at the starting day of the experiment (t_1) (t_1=0) and at any given day of the experiment (t_2).

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. Total protein content was measured according the method of Lowry (1951), with BSA as a standard. Total carbohydrates were quantified as glucose equivalents by the phenol-sulfuric acid method (DuBois et al., 1956). 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 (1978).

All spectrophotometrical analysis were performed using a T70 UV/Vis (PG INSTRUMENTS LTD, Leicester, UK).

The viscosity of the culture supernatant shows the amount of extracellular polysaccharide synthesized. It was measured by a viscosimeter B3 (VEB MLW, DDR). The algal cultures were centrifuged for 30 min. at 8000 g. The viscosity was reported by the following formula: η= t x (Q1-Q2) x K, where η is the dynamic viscosity of the liquid (mPa.s); t - time for the ball to fall in the viscosimeter; Q_1- ball density; Q_2-density of the studied liquid; K- ball constant (m $Pa.cm^3/g$). 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 metter (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). Values of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Figure 1 shows that medium 1 (M1) stimulated the biomass accumulation better, regardless of the CO_2 concentration. The highest yield achieved was 4.0±0.1 g L^{-1}. When the culture was supplied with 0.04% CO_2 only, it grew exponentially, but slower with a maximal yield of 3.4±0.1 g L^{-1}. On medium 2 (M2), *Porphyridium cruentum* had a more rapid exponential growth than M1 during the first two days. On the 48th hour, the better growth of the culture supplied with 2% CO_2 started to get noticable. It made a huge leap in growth between second and third day, ([μ]=0.677), but then the growth rate slowed down. Both cultures, cultivated on M2, entered stationary phase on the fifth day. *Rhodella rheticulata* (Rhodophyta) grown under the same cultivation conditions, reached maximal yield of about 2.5 g L^{-1} for 96h (4 days), which is in the same range as our strain of *Porphyridium cruentrum* (Ivanova et al., 2015). However, as stated above, the maximal yield of *P. cruentum* was much higher.

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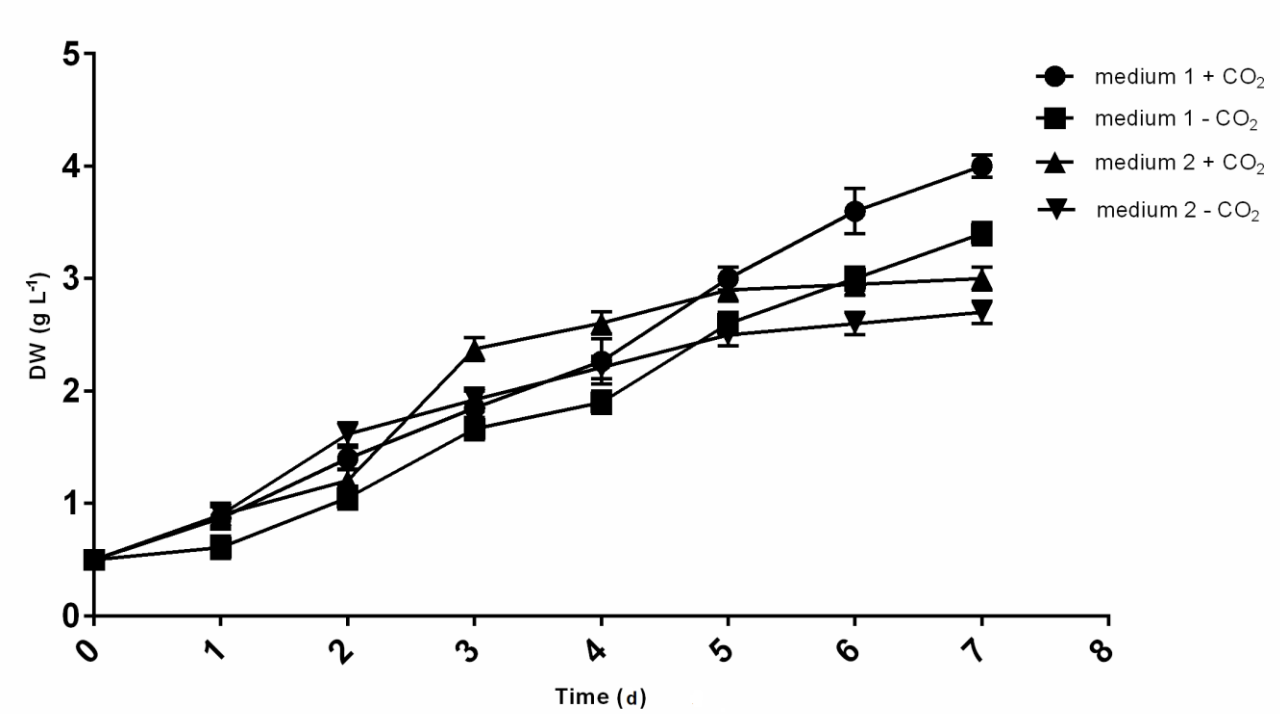
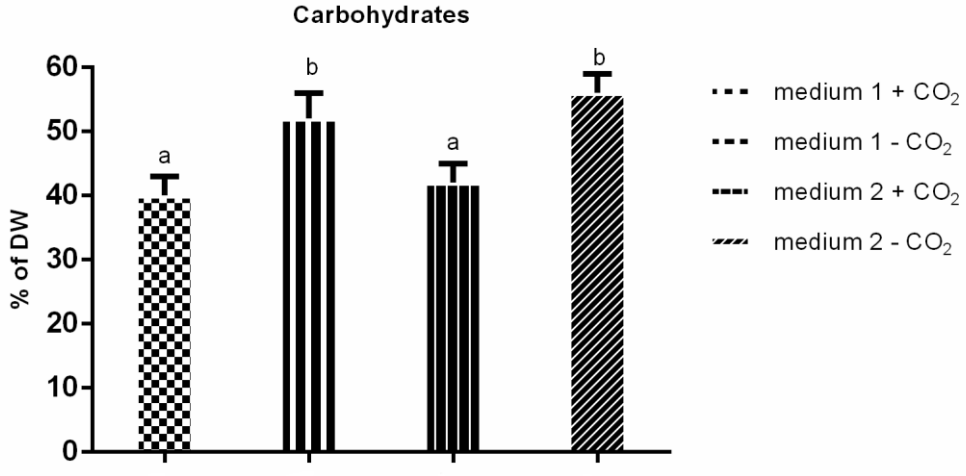


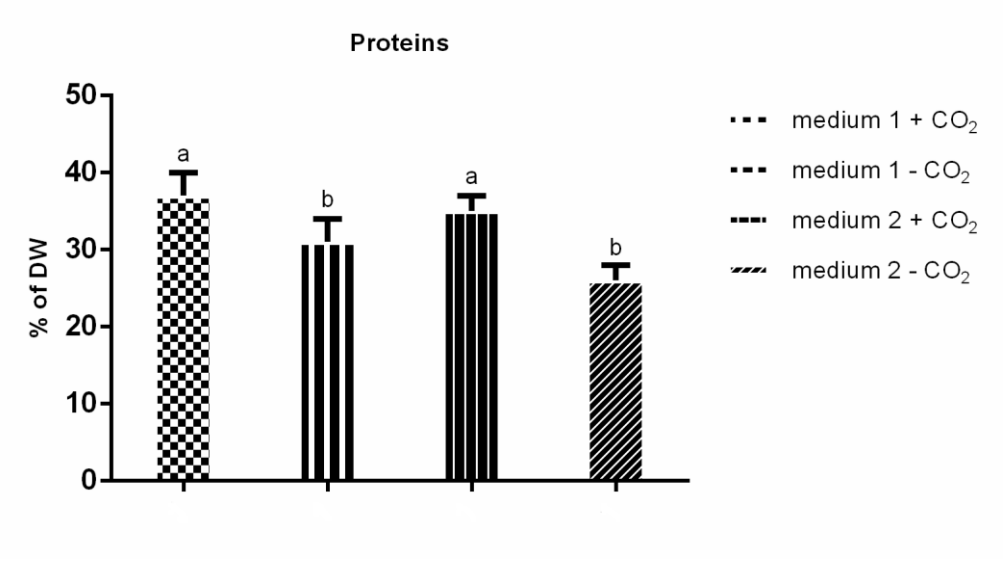
Fig. 1. Growth curves of *Porphyridium cruentum* depending on the nutrient medium composition and the CO_2 concentration.

In addition to the necessity to maintain a stable growth rate, it is also important for the algae to keep up a balanced composition of the cellular biochemical components.

Porphyridium cruentum did not show significant deviation in the biochemical composition during the different treatments. The highest was the carbohydrate content - up to 56% of DW, followed by the proteins (26-37% of the DW); and lipids (6-7% of DW); (Figure 2). Figure 2 clearly shows that the amount of carbohydrates and proteins depends on the CO_2 concentration and not as much on the nutrient content. Low CO_2 concentrations induced carbohydrate synthesis at the expense of proteins. The amount of lipids was very low and remained the same ($P > 0.05$), regardless of the cultivation conditions. Nitrogen limitation in the microalgal growth media has been previously reported to induce high accumulation of carbohydrates or lipids (Kim et al., 2017). In this investigation, the lower concentration of nitrates in M2 caused an increase in the carbohydrate production. *Porphyridium cruentum* (S.F. Gray) Nägeli, cultivated on a medium with nitrates as a nitrogen source (del Pilar Sánchez-Saavedra et al., 2018), had lipid content of about 8-10% of DW, and carbohydrate content - around 40% of DW when the culture was grown under lower light intensity. These values were confirmed in our study when cultivating the alga on M1 with sufficient amount of nitrates.



A



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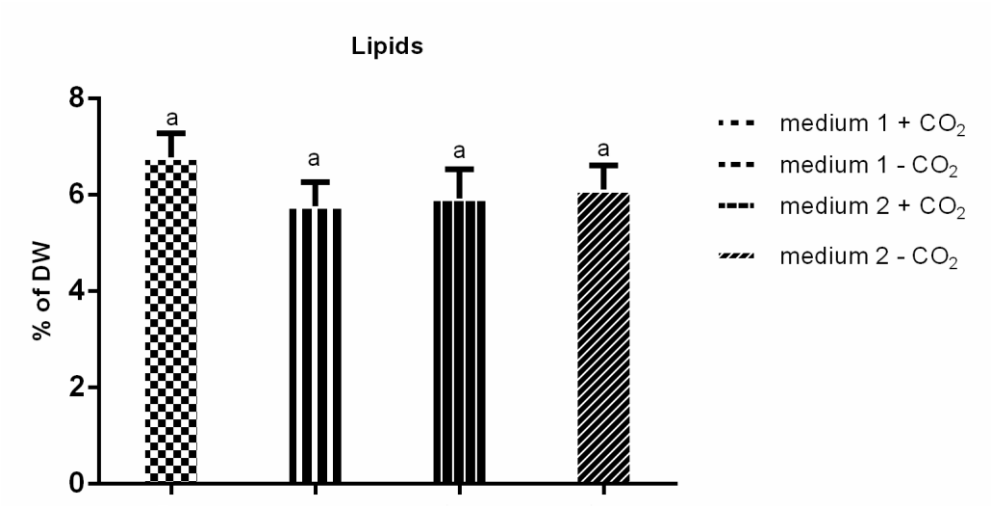
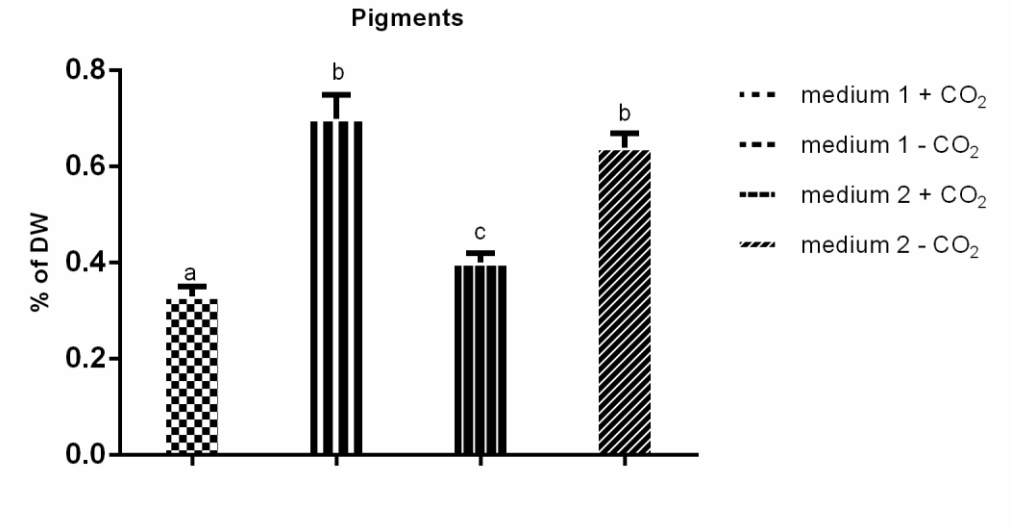


C



D

Fig. 2. Carbohydrate (A), protein (B), lipid (C) and pigment (chlorophyll+carotenoid) (D) content obtained from cultivation of *Porphyridium cruentum* in different nutrient media and at different concentrations of CO_2. Different lower case letters indicate significant differences for each parameter ($P < 0.05$).

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Tab. 1.

Phycobiliprotein content in the biomass of *Porphyridium cruentum* cultivated in different nutrient media and at different concentrations of CO_2.

Phycobilliproteins(% DW)	Medium1+CO_2	Medium1-CO_2	Medium 2 +CO_2	Medium 2-CO_2
Phycoerythrin	0.73±0.11	1.55±0.33	1.1±0.1	1.6±0.2
Phycocyanine	0.14±0.02	0.18±0.02	0.22±0.01	0.202±0.01
Alophycocyanine	0.13±0.02	0.32±0.06	0.36±0.02	0.269±0.02
Total	1±0.07 **a**	2.05±0.08 **b**	1.65±0.07 **c**	2.05±0.17 **b**

*Different lower case letters indicate significant differences for each parameter (P <0.05).

Since it is a red microalgae, its chlorophylls and carotenoids were in very small amounts. Red microalgae typically contain small amounts of cholorophylls and carotenoids, and the same applies fo *Porphyridium cruentum* (Figure 2D). That is not the case with the phycobilioproteins, though. From the results on Figure 2D and Table 1 was revealed a certain tendency. Higher amount of pigments (eiher chlorophylls+carotenoids, or phycobiliproteins) were accumulated when the cultures were supplied with 0.04% CO_2, and there were no significant differences between the produced quantities (P<0.05) on M1 and M2. Growing *Porphyridium cruentum* on M2 provided a better pigment yield. This makes the medium more suitable for the production of these metabolites. The reason for that could be attributed to the amount of Cl^- in the nutrient media. Salt concentration up to 0.6 M resulted in an elevation of the total phycobiliprotein content in *Spirulina* spp., while a further increase of the concentration up to 0.9 M affected negatively the phycobiliprotein synthesis (Hifney et al., 2013).

Data for polysaccharide viscosity obtained during the stationary phase are presented in Table 2. The initial viscosity was 1.8±0.1 mPa.s. Growing *Porphyridium cruentum* in M1 influenced possitively the polysaccharide synthesis. In combination with 2% CO_2, this medium provided the best conditions for high productivity of the component. Han et al. (2017) revealed the importance of the enzymes PGI and FBP-ase in stimulation of the biosynthesis of *N. flagelliforme* exopolysaccharides. Structural and catalytic chemistry of FBP-ase requires metal ions for catalysis - Mg^{2+}. In our research,in M1 the concentration of Mg^{2+} was a few times higher than in M2 which probably contributed to the stimulation of the polysaccharide synthesis.

Tab. 2.

Viscosity of polysaccharide obtained from *Porphyridium cruentum* cultivated in different nutrient media and at different concentrations of CO_2.

Media	**Viscosity (mPa.s)**
Medium 1+CO2	6.3±0.3 **a**
Medium 1-CO2	4.8±0.2 **b**
Medium 2+CO2	3.2±0.2 **c**
Medium 2-CO2	4.0±0.2 **d**

*Different lower case letters indicate significant differences for each parameter (P <0.05).

Low concentration of CO_2 reduced the production of polysaccharides by about 25% in M1. Compared to M1+CO_2, the productivity of M2+CO_2 was 2 times lower (Table 2). M2 supplied with atmospheric air had lower polysaccharide productivity than M1+CO2, but better than when the algae were cultivated in M2+CO_2. The obtained data for M2 confirmed the results of Li et al. (2000) who indicated that the formation of the soluble fraction of the cell-wall polysaccharide in the medium was higher in the air-supplied culture in comparison to a CO_2-enriched culture, both on a per cell basis. These results suggest that CO_2 concentration affects polysaccharide composition by altering the partitioning of the fixed carbon, likely by modification of the biosynthetic pathway (Li et al., 2000). Probably, the polysaccharide is a prevalent product predominantly synthesized to balance the overall cellular metabolism when fixed carbon is available in excess within the cell (Rai, 1995).

As *Porphyridium cruentium* is a slowgrowing alga, the pH of the medium also changed slowly. The cultures supplied with 2% CO_2 maintained their pH neutral during the whole cultivation. The pH of the cultures supplied with 0.04% CO_2 was 8.5 and 8.8 for medium 1 and medium 2, respectively. These values are consistent with the normal algal physiology. In a study on *Porphyridium cruentum* Naegeli, the concentration of CO_2 and pH of its nutrient medium affected both the growth rate and the accumulation of exopolysaccharide (Lutzu et al., 2016). When the cultures were grown with atmospheric air, they had high growth rate, even higher when the cultures were aerated with 2% CO_2. Even so, the accumulated exopolysaccharides were in lower quantities when the concentration of CO_2 was lower as well. Also, pH values, closer to neutral, influenced positively the accumulation of exopolysaccharides than values of 8.5, which was again confirmed by our investigation (Table 2).

CONCLUSIONS

Porphyridium cruentum (AG.) NAG Vischer 1935/107 has proven to possess very good adaptive mechanisms as it grew well on both studied nutrient media. Depending on the biotechnological needs, M1 is suitable for achieving high biomass yield, and in combination with 2% CO_2 aeration produces high amount of exopolysaccharides. M2 is more suitable for obtaining higher quantities of pigments. The lower CO_2 concentrations have a potential in higher pigment production. The data can be used for introducing the biotechnological advantages of the alga as a producer of valuable metabolites for a broad range of industries.

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