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Optimization strategies for improved growth, polysaccharide production and storage of the red microalga *Rhodella reticulata*

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To ensure good conditions for growth and heteropolysaccharide production by the red microalga *Rhodella reticulata*, optimization of the medium and conditions was performed. Stimulation of the growth of microalgal cells up to1.6-fold was achieved with a new medium. Maximal growth rate (μ=0.19 d^{-1}) was reached at the beginning of stationary phase of growth (72 h), together with the increase in the pigment content. The influence of the great quantity of humic acids and thiamin in the medium (proved by HPLC) on algal growth was also established. The amount of extracellular polysaccharide (0.365 mg ml^{-1}) produced by the algal cells cultivated in the new medium was 1.4-fold higher compared to the quantity in the standard medium. The immobilization of *Rhodella reticulata* cells into super-macroporous cryogel of 2-hydroxyethylcellulose lead to a prolonged period of storage. Algal cells were vital after three months and could be successfully used for the production of extracellular polysaccharide with values for immobilized cells 1.3 –fold higher than those for free cells.

Key words: Algae; Bioactive substances; Growth medium; Immobilization

INTRODUCTION

A successful biotechnological process depends on the selection of a suitable organism capable to produce a desired product at optimal conditions. By 2015, the industrial biotechnology will have a 20% share in total chemical production [1]. The red microalgae of the genus *Rhodophyta* are potential sources of unique bioactive substances [2-3] that can find different applications - in medicine, pharmacy, cosmetics, food industry as food supplements, etc. [4-5]. Typical representative of the genus is the unicellular red microalga *Rhodella reticulata*, which has the ability to synthesize and release part of the polysaccharide material into the culture medium [6-7]. Its functions are mainly protective but its antiviral and antitumor effects are well known [8-9]. The growth of microalgae is primarily affected by abiotic environmental factors. Some biologically active organic compounds, namely vitamins, nucleic acids and organic matter such as humic substances and soil extracts, also stimulate the growth of algal cultures [10]. However, long cultivation of algae in a laboratory usually leads to contamination and decreased growth rates and densities, when cultured in traditional media [11]. It is suggested that the type of nutrient medium plays a critical role for the algal growth [12]. Therefore, the suitable balancing of the elemental composition of the growth medium is a tool to obtain high-density cultures. Different optimization strategies were applied to the media components in order to increase the productivity of algal cells, pigment synthesis and polysaccharide production [13-14]. An approach to keep the vitality of cells after long-term storage of the algal cultures in the laboratory is the immobilization technique. It is based on finding suitable carriers that can ensure preservation of the algal cultures for a long period under laboratory conditions. Immobilization of different algae had already been performed in alginate beads to increase the period of storage and preserve their physiological activity [15]. At the same time, immobilization facilitates both the extraction of the extracellular polysaccharide from the medium and the handling of the system [16].

Super-macroporous polymer cryogels are an interesting class of materials due to their unique heterogeneous open porous structure, which significantly increases the equilibrium sorption properties and allows unhindered diffusion of solutes, nano-particles and micro-particles. Usually, cryogels possess spongy-like structure of huge pores (50-200 μm) containing free water surrounded by thin walls and, therefore, they are often used for immobilization

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of enzymes and cells by entrapment inside the channels of interconnected pores [17].

The aim of the present study was to find the most appropriate conditions for intensive algal growth and increased polysaccharide production of *Rhodella reticulata* by optimizing the components of the nutrient medium and to prolong the storage of the algal culture with the immobilization technique.

EXPERIMENTAL

The red microalga *Rhodella reticulata (Rhodophyta)*, strain UTEX LB2320 was acquired from the Austin University, Texas, USA. It was isolated from brackish water in the region. It is maintained as a working culture in the laboratory collection of the Department of Experimental Algology of the Institute of Plant Physiology-Bulgarian Academy of Sciences.

Intensive cultivation

To test the effect of culture media and polysaccharide production of free and immobilized algal cells, all culture experiments were conducted at permanent illumination of 150 μE m^{-2} s^{-1} in special vessels (100 ml) for intensive cultivation and 2% CO_2 supply. Temperature was maintained at 29 - 30°C and pH = 7.3. The algae were grown in 5 nutrient media.

Soil extract preparation

The soil was collected from a region with pine trees. Air-dried soil and twice its volume of distilled water were autoclaved together at 15 psi for 15 min. After centrifugation at 3000 g, the supernatant was decanted and then filtered to a final volume of 100 ml.

Dry weight measurement

The growth of the algal culture was estimated following the increase in its weight. For this purpose, 10 ml of the algal suspension were centrifuged at 6000 g for 20 min (Rotofix 32A, Hettich). The supernatant was removed and the cells were dried at 105°C for 16 h. The salts were eliminated by rinsing thrice with tap water. Cell count to evaluate the growth and development of the investigated strain was carried out by using a Burker counting chamber.

Specific growth rate (μ, d^{-1})

Specific growth rate was calculated from the dry weight: μ=ln (N2/N1)/t2-t1, where N1 and N2 are the dry weights of algal cells at definite times t_1 and t_2.

Pigment content

It was estimated spectrophotometrically. The amounts of chlorophyll "a", carotenoids and phycobiliproteins were given according to the absorption spectra, taking into account the absorbances at: 665 nm for chlorophyll "a", 460 nm for carotenoids, 565 nm for phycoerithrin and 620-650 nm for phycocyanin. Pigments content was determined from the absolutely dry weight, employing the equations of Siegelman and Kucia [18].

ICP-OES analysis

The elemental composition in the soil extract was determined by inductively coupled plasma atomic emission spectroscopy (Varian-Vista MPX CCD Simultaneous ICP-OES).

Carbohydrates analysis

The extracellular carbohydrates were analyzed by the phenol-sulphuric method of Dubois [19] using glucose as a standard.

Organic carbon analysis

The determination of soil organic carbon was based on the Walkley-Black chromic acid wet oxidation method. Oxidation matter in the soil is oxidized by 1 N $K_2Cr_2O_7$ solution. The remaining dichromate is titrated with ferrous sulphate. The titre is inversely related to the amount of carbon present in the soil sample [20].

HPLC analysis

The amounts of humic acid and thiamine were determined using a modular HPLC system consisting of a pump Perkin Elmer, Series 10, Liquid chromatograph, chromatography column C18, UV-detector. Mobile phase 9:1, phosphate buffer: methanol, pH = 3, flow rate 1ml min^{-1}.

Matrix synthesis

HEC cryogels were synthesized by a procedure described elsewhere [21]. Briefly, 2 mass % aqueous solution of HEC (Hercules Inc.; MW 1 300 000 g/mol) and photoinitiator ((4-benzoylbenzyl) trimethylammonium chloride, 2 mass% to HEC) were poured into Teflon dishes (20 mm diameter) forming a 2.5 mm thick layer and kept in a freezer at minus 20 °C for 2 h. The frozen system was irradiated with full spectrum UV-vis light with a ''Dymax 5000-EC'' UV curing equipment with 400 W metal halide flood lamp for 2 min (dose = 11.4 J/cm^2; input power = 93 mW/cm^2). Finally, cryogels were extracted with distilled water and freeze dried. Gel fraction yield = 92 ± 2%.

During cell entrapment the algal suspension was centrifuged and re-suspended, as mentioned above, to

reach a concentration of 5.10^6 cells ml^{-1}. The freeze dried matrices were immersed into the suspension and left under illumination at 25°C without CO_2 supply for 10 days until the whole surface was abundantly covered with algal cells. Cell count was accomplished by mechanical disruption of one matrix with cells. The experiment was carried out in triplicate and average values were presented.

For each medium the experiments were carried out in 2 replicate flasks and every experiment was repeated 3 times. Values presented in the figures are the means ± standard deviations of the different experiments.

RESULTS AND DISCUSSION

Growth, pigment content and polysaccharide production in different media

The growth and development of *Rhodella reticulata* were studied in five different media to estimate their influence. Media constituents are presented in Tables 1 and 2.

The initial cell dry weight employed in the experiments was 0.85 mg ml^{-1} and its change was measured for a period of 72 h (Fig. 1). Medium 5, we will call medium C for convenience. The obtained results after comparison of the 5 different media showed that the most intensive growth of cells was observed for the new medium C.

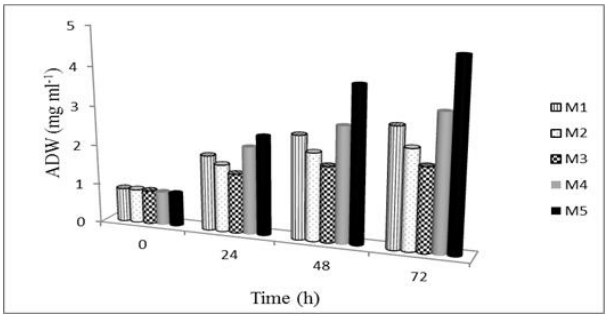

Fig. 1. Development of *Rhodella reticulata* for 72 h using 5 different media. (M-medium)

The optimization of the concentration of the participating nutrients and the addition of soil extract resulted in important quantitative and qualitative differences from the other media used. The growth was worst in medium 3 and medium 2 and best in medium C, in which the algal growth was increased 1.6-fold at the 72^{nd} h compared to the standard medium 1. The new medium C contained 1076 mg l^{-1} nitrogen.

Table 1. Compositions of 5 different types of media used for the cultivation of *Rhodella reticulata*

Media components, (mg l^{-1})	Type of medium				
	1	**2**	**3**	**4**	**5**
KNO_3	1240	-	-	620	826
$NaNO_3$	-	1250	750	375	250
$CaCl_2.2H_20$	-	2.5	2.5	1.25	0.83
KH_2PO_4	-	175	175	87.5	58.3
K_2HPO_4	620	75	75	347.5	413.3
$MgSO_4.7H_2O$	2500	75	75	1287.5	1692
KCl	8000	-	-	4000	5333
NaCl	6260	6260	6260	6260	6260
KI	500	-	-	250	333
KBr	500	-	-	250	333
ME	0.72	0.9	0.9	0.40	0.51
$Ca(NO_3)_2$	170	-	-	85	113
Vitamin B12			$5\text{-}10.10^6$ g l^{-1}		
Soil (soil extract)	-	30000	30000	15000	10000
EDTA	9.3	80	80	45	33
$FeSO_4.7H_20$	-	4.98	4.98	2.49	1.66
H_3BO_3	1.55	11.42	11.42	6.485	4.83
% salinity	0.6	0.6	0.6	0.6	0.6

1. Medium of Pekarkova; 2. Medium of Bold (1985) with 3-fold increase in the nitrogen content and soil extract; 3. Medium of Bold with 2-fold increase in the nitrogen content and soil extract; 4. Combination of (medium 1) + (medium 2) in a ratio of 1:1; 5. Medium C - combination of (medium1) + (medium 2) in a ratio of 2:1.

Table 2. Microelements composition in the Pekarkova, Bold and C media

Trace elements (mg l^{-1})	Pekarkova medium	Bold's Basal medium	Medium C
H_3BO_3	3.09	11.4	7.76
$MnSO_4.4H_2O$	1.2	1.44	1.52
$CoSO_4.7H_2O$	1.4	0.49	1/17
$CuSO_4.5H_2O$	1.24	1.57	1.62
$ZnSO_4.7H_2O$	1.43	8.82	5.36
$NH_4Mo_7O_{24}$	1.84	0.71	1.59

In comparison to the standard medium 1, the nitrogen quantity was lower. Although the nitrogen content in medium 2 was increased (1250 mg l^{-1}), no intensification of growth of *Rhodella reticulata* was registered.

This result showed that nitrogen concentration in the different variants of the medium (about 1000 mg l^{-1}) is enough for the culture growth and further addition of nitrogen did not lead to an increase in algal growth. Mn, Cu, B, Zn and Co in trace amounts are capable of inducing the growth of microalgae; at the same time, higher concentrations of these micronutrients hinder the growth of microalgae [22, 23]. The new improved medium C, possessed optimal amounts of these micronutrients which resulted in maximum growth. As cells entered the stationary phase of growth (day 4), medium C provided the highest specific growth rate - 0.19 d^{-1} (Fig 2). We assume that the highest increase in the algal growth in medium C is probably due to the most suitable balance between the basic components. Approximately the same level of growth rate in a medium specific for *Haematococcus pluvialis* was obtained in an airlift reactor reported by Kaewpintong *et al*.[24].

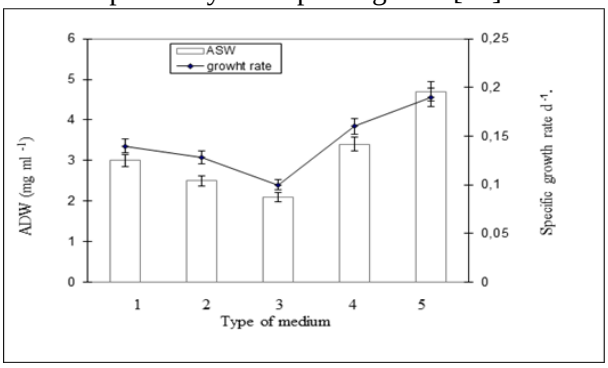

Fig. 2. Absolutely dry weight and specific growth rate of *Rhodella reticulata* obtained during cultivation in the various types of media tested.

Analysis of the elemental composition of the media tested

The main factor for enhanced growth and increase in pigment content and polysaccharide production appeared to be the choice of the basic components in the medium, together with their most suitable balance. The quantity of some constituents in the combined medium C was reduced as follows: KI, KBr - by 170 mg, $MgSO_4$ - by100 mg. The quantity of EDTA was increased 3-fold in the medium C. Important appeared to be the contribution of trace elements in the media (Table 2). Since medium C was based on Pekarkova and Bold's media, a comparison of the trace elements contained in both media showed that the only significant difference was the 6 - fold greater amount of $ZnSO_4$ and the 3.8 - fold greater amount of H_3BO_3 in the medium. Thus, the Zn concentration seemed to contribute for better growth in the new medium as compared to the standard one.

Pigment content of algae is an important factor that reveals the state of the algal culture. The comparison of the results for pigment quantity at the 72th h of *Rhodella*'s development was carried out. The quantity of pigments was calculated for the medium C and the standard medium. The results showed an increase in the whole pigment quantity by about 1.35-fold compared to the control (Table 3). The pigment content in microalgae is a specific feature for each species. Its evaluation is essential as an indirect measure of cell growth [25]. On the other hand, the pigments can serve as a valuable bioproduct used in different applications: medicine, cosmetics and food industry [26]. In the course of the experiments the production of exopolysaccharide was also followed in the new medium C. The standard "Pekarkova" medium was tested as a control. The polysaccharide production at intensive cultivation was followed with initial algal cell quantity of 1.10^6 cells ml^{-1} for 120 h (Fig. 3).

The direct comparison of the results obtained for the standard growth medium used for *Rhodella reticulata* cultivation and the new formulation revealed a more intensive growth of algal cells, increased pigment quantity and higher polysaccharide production.

Table 3. Percent content of pigments from the absolutely dry weight (ADW)

Pigments type	Pigments content (% from ADW)	
	Pekarkova medium	Medium C
Carotenoids	0.46	0.61
Chlorophyll "a"	0.39	0.52
Sum of phycobiliproteins	10.1	13.6

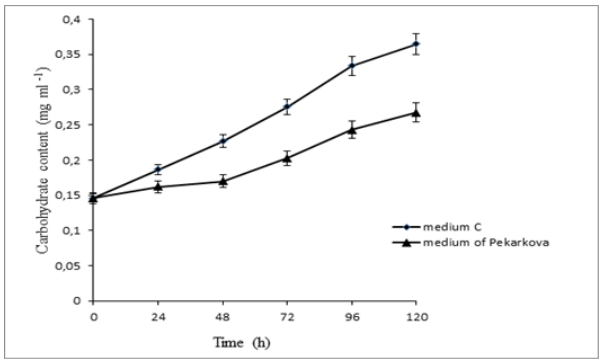

Fig. 3. Production of extracellular polysaccharide by *Rhodella reticulata* cultivated in two media (Pekarkova medium and medium C).

Starting from an equal quantity of polysaccharide in the beginning of the process, at the 120th h, the difference between the carbohydrate contents using the two different media reached about 1.4 - fold. The use of the new medium C led to obtaining of higher values of the polysaccharide quantity - 0.365 mg ml^{-1}, towards 0.267 mg ml^{-1} for the standard one. Finding the optimal conditions and medium composition for the increased biosynthesis of algal polysaccharides will permit this valuable product to be used in its many applications [27]. This is achieved by the use of the new medium C. This fact could be a prerequisite for a large-scale production of this biopolymer.

Soil extract had been reported to promote the highest increase in dry weight of the marine microalgae *Ulva lactuca* [28]. *Cladophora glomerata,* an attached green alga is a species that grows well on synthetic media supplemented with soil. The same fact was evident from our experiments with *Rhodella*. The exact constituents in the soil extract as an important part of the nutrient medium become essential for the implementation of different physiological experiments [29]. We suggest that the presence of soil extract in the new medium is another factor for growth intensification, which we analyzed precisely. The results did not reveal significant differences in the elemental inorganic constituents of the standard and the new medium, where soil extract was present. Evaluation of the constituents in the soil extract was carried out using ICP-OES analysis (Table 4).

Another survey followed the organic components available in the soil extract. Using the HPLC method and Black chromic acid wet oxidation, the presence of humic acids and thiamine was proved. Their stimulating effect was established by other researchers as well [30].

Table 4. Elemental composition in the aqueous soil extract

N	Elemental composition	Content (mg l^{-1})
1	Aluminum	0.2
2	Arsenic	<0.5
3	Calcium	112.8
4	Cadmium	0.011
5	Cobalt	0.015
6	Copper	0.075
7	Iron	0.56
8	Potassium	61.7
9	Magnesium	24.0
10	Manganese	0.555
11	Sodium	28.7
12	Nickel	0.046
13	Phosphorus	9,6
14	Lead	<0.03
15	Sulfur	20.5
16	Zinc	0.743

The values for the quantity of humic acids and thiamine are presented in Figure 4. Retention time of humic acids was 2.435 min and retention time of thiamine was 4.468 min. The amounts of thiamine and humic acids introduced in the new medium C were 40 mg and 85 mg, respectively, obtained from 10 g soil. They are about 30% from the soil organic carbon content. The quantity of organic carbon is about 2.9 % of the soil.

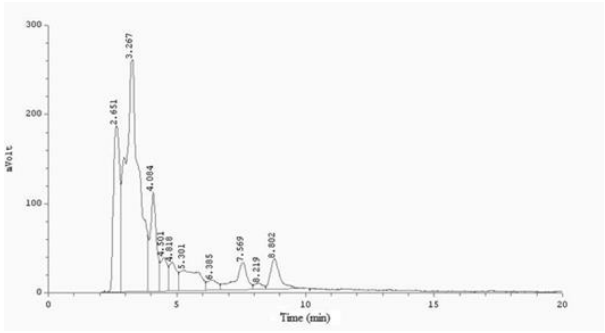

Fig. 4. HPLC determination of thiamine and humic acids content.

Following the HPLC and the ICP-OES analyses, we found that the most important difference of the new medium from the other ones was the presence of thiamine and humic acids. That was the reason for substituting the soil extract with these two components in quantities comparable to those already measured. They appeared to play a major role for the algal growth as organic constituents in the medium with soil extract. To test their effect on algal growth, 3 variants of media were involved, where in addition to the control – standard medium, variant 2 - medium C with humic acids and thiamine, quantity as obtained from the HPLC analysis and 3 - medium C with replaced humic acids and thiamine with soil extract in the same quantity. The results proved that growth was stimulated to the same extent as in the presence of soil extract (Fig. 5).

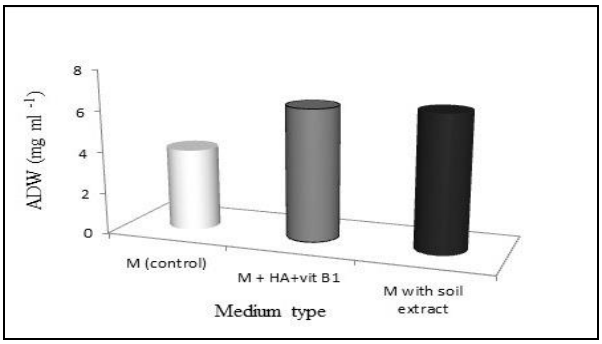

Fig. 5. Influence of humic acids and vitamin B1 on the growth (ADW) of *Rhodella reticulata*

The results obtained proved the significant role of these two organic constituents present together in the medium. When soil extract was included, the values for ADW were 6.55 mg ml^{-1} compared to 6.4 mg ml^{-1} when HA and vitamin B1 were added. Literature data also showed that the humic acids and thiamine are capable of enhancing algal production and quality. Only 80-100 mg l^{-1} of humic acids are required to greatly increase pigment development and growth, influencing the metabolism by their chelating activity [31]. The presence of thiamine is also of major importance for the growth and development of algae. Vitamin B1 is important for primary carbohydrate and amino acid metabolism and likely useful as an anti-oxidant [32]. Depending on the specific conditions, both types of the media (variants 2 and 3) could be appropriate for algae cultivation. If the purpose of the experiment is proving physiological or biochemical characteristics, variant 2 will be more suitable. When we pursue mass cultivation and production of red algae, variant 3 has to be used. In that way the cost of production will be decreased and the biomass yield will be increased. Employing medium C led to significantly higher polysaccharide production and higher pigment content correlated with more intensive growth of the algal strain. As a result from the changes in the nutrient media, during the abundant growth of the algal culture the regulation in its metabolic activity was changed, concerning the pigment and extracellular polysaccharide synthesis.

Cell immobilization

The red microalga *Rhodella reticulata* was cultivated and entrapped for the first time in a HEC cryogel matrix and tested for long-term storage and exopolysaccharide synthesis. The immobilized algae were let to grow on to the polymer matrix for 10 days and then, the cell count was performed. The pure HEC matrix and the matrix with immobilized algal cells are visualized on the digital image (Fig. 6).

Fig. 6. Digital image of the matrix without cells (left) and the matrix with entrapped cells (right)

The good growth and development of the algal culture proved the appropriate choice of matrix for *Rhodella reticulata*. The quantity of immobilized cells was 5.3 10^6 cells ml^{-1}, which is similar to the free cells cultivation. This result indicates that HEC matrix provides suitable conditions for algal cells growth. In the next step pieces of matrix with immobilized algal cells were transferred to the new medium C and left to develop in flasks under extensive conditions of growth for a period of 15 days. During this period the immobilized *Rhodella* cells started to produce extracellular polysaccharide.

The results showed that starting from a lack of polysaccharide in the beginning because the matrices with cells were transferred into fresh medium, the difference of the carbohydrate content, using free and immobilized cells appeared to be about 1.3-fold at the 20^{th} day. The use of immobilization procedure led to higher values of the exopolysaccharide quantity (Table 5).

The conditions for long-term storage of algal cultures in a laboratory aim to preserve culture vitality. The algal cells immobilized into HEC cryogels were still alive after 90 days. This phenomenon can be attributed to the presence of free water in the macroscopic pores of the cryogel that preserves cells vitality. The survival of immobilized cells after three months storage in a refrigerator in absolute darkness at 4°C without nutrient medium was tested in 30 ml of the new medium C. The estimation of the cell growth was carried out by applying cell count.

Table 5. Content of extracellular polysaccharide produced by immobilized and free algal cells at intensive cultivation.

Time (days)	Polysaccharide (mg ml^{-1})	
	Immobilized cells (1.5 10^6 cells ml^{-1})	Free cells (1.5 10^6 cells ml^{-1})
0	0.0	0.15
24	0.09	0.18
48	0.16	0.19
72	0.25	0.21
96	0.29	0.23
120	0.41	0.26

The matrices were disrupted and immersed into the medium and put at extensive cultivation for 20 days. The initial cell number was 4.6 10^6. The number of cells appeared to be 18.4 10^6 in 30 ml after 20 days, showing the good development of the algal culture.

Immobilization of the red microalga into 2-hydroxyethylcellulose cryogel was performed for the first time. The immobilized cells revealed significantly higher polysaccharide production and more intensive growth. The cells immobilization also led to a prolonged period of storage of vital cells and enhanced production of the desired product, the polysaccharide. The increased quantity of polysaccharide produced was probably due to the new medium that permits good growth and development, and on the other hand, to the suitable conditions provided by the immobilization for the cells. The separation of polysaccharide from the medium was much facilitated when the cells were in an immobilized form. We have to note that the cryogel matrices were synthesized from a low-cost polymer from renewable sources employing a very facile method. Thus, the utilization of HEC cryogels for immobilization and long-term storage of algal cell appears to be an efficient and cheap approach to keep the cells vital and, on the other hand, eliminate the risk of contamination at frequent sifting of the algal cultures.

The isolated polysaccharide was subjected to purification and lyophilization. This ready product is being tested at the moment as a supplement in natural cosmetic products such as gels, creams and serums (data not shown).

CONCLUSION

Algae could significantly contribute to industrial biotechnology. Our experiments proved the increased algal growth by the addition of soil extract to the new medium C as a cheap way for reaching mass production of red algae. Microalgal cultures are recently receiving much attention because of the biotechnological and biomedical production of active biomolecules. A new technological scheme employing immobilization was developed that increased the algal growth and polysaccharide production. For most of the applications, the market is still developing and the biotechnological use of microalgae will extend into new areas.

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СТРАТЕГИИ ЗА ОПТИМИЗАЦИЯ И УВЕЛИЧАВАНЕ НА РАСТЕЖА, ПРОДУКЦИЯТА НА ПОЛИЗАХАРИД И СЪХРАНЕНИЕ НА ЧЕРВЕНОТО ВОДОРАСЛО *Rhodella reticulata*

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(Резюме)

За да се осигурят добри условия за растеж и продукция на хетерополизахарид от червеното микроводорасло *Rhodella reticulata*, е осъществена оптимизация на средата и условията. При използването на нова среда е постигната стимулация на растежа на микроводорасловите клетки до 1.6 пъти. Максимална скорост на растеж (μ=0.19 d^{-1}) е достигната в началото на стационарната фаза на растеж (72 h), заедно с увеличаване на пигментното съдържание. Установено е влиянието на голямото количество хуминови киселини и тиамин в средата (доказано с HPLC), върху растежа на водораслото. Получените стойности за количеството екстрацелуларен полизахарид (0.365 mg ml^{-1}), продуциран от водорасловите клетки, култивирани в новата среда са 1.4 пъти по-високи в сравнение количеството при използване на стандартна среда. Имобилизацията на клетките на *Rhodella reticulata* в супер-макропорьозния криогел на основата на 2-хидроксиетилцелулоза доведе то удължителен период на съхранение. Водорасловите клетки са запазили жизнеността си след три месеца и могат успешно да бъдат използвани за продукция на екстрацелуларен полизахарид, като стойностите за количеството му са 1.3 пъти по-високи в сравнение с количеството, синтезирано от свободни клетки.