Avanços Científicos e Tecnológicos em Bioprocessos

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(Organizador)

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APRESENTAÇÃO

Avanços Científicos e Tecnológicos em Bioprocessos é uma obra que reúne vinte e três capítulos com temas em pesquisas científicas realizadas no campo da biotecnologia, e que envolve agentes biológicos e bioquímicos na geração de produtos ou processos. Nesta obra se concentram diversos avanços descritos nas metodologias e nos resultados, distribuídos em quatro tópicos principais, envolvendo: processos químicos e biotecnológicos no aproveitamento de resíduos; produção de metabólitos e enzimas; métodos analíticos e de simulação; e biotratamentos envolvidos na geração de energias. Esta obra foi escrita por jovens pesquisadores brasileiros que estão desenvolvendo suas teses e/ou dissertações em instituições nacionais. Por este motivo, os aspectos inovadores e o alcance dos resultados apresentados podem ser um grande estímulo para aqueles que visam conhecer com maior amplitude alguns dos aspectos biotecnológicos estudados em algumas das instituições de nosso país.

Alberdan Silva Santos

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CAPÍTULO 23

EFFECT OF TEMPERATURE AND SALINITY ON THE PRODUCTION OF CAROTENOIDS AND LIPIDS BY MARINE MICROALGA

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RESUMO: Esta pesquisa identificou os carotenoides presentes no extrato de *Dunaliella tertiolecta* e avaliou os efeitos da temperatura e da salinidade do meio na composição elementar e nos teores de lipídios e carotenoides desta microalga marinha cultivada em um fotobiorreator airlift. Os experimentos foram realizados de acordo com um delineamento composto central rotacional (DCCR). Seis carotenoides foram identificados por cromatografia líquida de alta eficiência (CLAE): *trans*-luteína, *trans*-β-caroteno, *trans*-zeaxantina, *trans*-neoxantina, *trans*-anteraxantina e 9-*cis*-β-caroteno. As condições ideais para a produção de carotenoides e lipídios por *D. tertiolecta* foram de 27 a 29 ° C e 0,7 a 0,8 M de NaCl. Nas condições testadas, as maiores quantidades de lipídios, luteína, β-caroteno e zeaxantina obtidas foram, respectivamente, 11,75 ± 1,8 %, 1315 ± 36 μg g^{-1}, 732 ± 51 μg g^{-1} and 244 ± 76 μg g^{-1}.

PALAVRAS-CHAVE: *Dunaliella tertiolecta*, airlift, carotenoides, lipídios.

ABSTRACT: This research identified the carotenoids present in *Dunaliella tertiolecta* extract and evaluated the effects of temperature and medium salinity on the elemental composition and lipid and carotenoid contents of this marine microalga cultured in a flat-panel airlift photobioreactor. The experiments were performed according to a central composite design (CCD). Six carotenoids were identified by high-performance liquid chromatography (HPLC): all-*trans*-lutein, all-*trans*-β-carotene, all-*trans*-zeaxanthin, all-*trans*-neoxanthin, all-

trans-antheraxanthin and 9-*cis*-β-carotene. The ideal conditions for carotenoid and lipid production by *D. tertiolecta* were 27 to 29 °C and 0.7 to 0.8 M NaCl. Under test conditions, the greatest amounts of lipid, lutein, β-carotene and zeaxanthin obtained were, respectively, 11.75 ± 1.8 %, 1315 ± 36 $\mu g\ g^{-1}$, 732 ± 51 $\mu g\ g^{-1}$ and 244 ± 76 $\mu g\ g^{-1}$.
KEYWORDS: *Dunaliella tertiolecta*, airlift, carotenoid, lipids.

1 I INTRODUCTION

The potential of microalgae as a commercial source of carotenoids and polyunsaturated fatty acids is widely recognised (Derner *et al.*, 2006). Carotenoids, such as β-carotene, lutein and zeaxanthin have a wide variety of market applications: natural food colouring agent; components in cosmetics and health foods; diagnostics and biomedical research (Francavilla *et al.*, 2010). In addition to their colouring properties, the carotenoids are known to have several others biological functions, including the enhancement of pro-vitamin A activity, cancer-preventing effects, cardiovascular disease protective effects and the reduction of cataract risks and age-related macular degeneration (Del Campo *et al.*, 2000; Raja *et al.*, 2007). Additionally, *Dunaliella* cells are rich in lipids and antioxidants, can be used as food supplement or food additive (Tafreshi and Shariati, 2009).

Large-scale production of microalgae bio-products is controlled by several environmental parameters as salinity, temperature, light intensity and nutrient availability (Baudelet *et al.*, 2017; Benavente-Valdés *et al.*, 2016). The use of closed bioreactors provides controlled and aseptic conditions for cultivation and efficient light utilisation, resulting in a consistent product quality and high cell densities (Raja *et al.*, 2007).

Dunaliella is a unicellular green alga (Chlorophyta, Chlorophyceae), found from mixed and euryhaline to hypersaline waters (Francavilla *et al.*, 2010). The salt tolerance of *Dunaliella tertiolecta* has been reported to be up to 3 M of NaCl (Jahnke and White, 2003).

In this paper, we report the effects of two key physicochemical variables, temperature and medium salinity (without interference from high concentrations of nutrients, such as nitrogen and carbon dioxide), on the production of lutein, β-carotene, zeaxanthin and total lipids by *D. tertiolecta*. From the analysis of the results, we report the optimal conditions for maximising the formation of bio-products. We present and discuss profiles of obtained carotenoids (all-*trans* forms of lutein, β-carotene, zeaxanthin, neoxanthin, antheraxanthin and 9-*cis*-β-carotene) as a function of growth conditions. Due to the expected increase in worldwide demand for these natural components, the findings of this study could lead to better industrial exploitation of *D. tertiolecta* biomass for carotenoid and lipid production.

2 I MATERIALS AND METHODS

2.1 Microorganism and Culture Medium

Marine microalgae *Dunaliella tertiolecta* BE 003 was obtained from the culture collection of the Department of Marine Biology, Federal University Fluminense (Niterói, Rio de Janeiro, Brazil) and maintained in f/2 medium (Guillard, 1975).

2.2 Photobioreactor Cultivation

The experiments were performed in flat-panel airlift photobioreactors (Kochem *et al.*, 2014). The aseptic photobioreactors were filled with 2.0 L of sterile f/2 medium and inoculated with 200 mL of algae pre-culture to a total 2.2 L working volume.

The cultures were performed in triplicate according to a central composite design at different temperatures (21 °C, 23 °C, 28 °C, 33 °C and 35 °C) and salinities (0.430 M, 0.513 M, 0.715 M, 0.917 M and 1.000 M). Different concentrations of NaCl were added to the medium to achieve the required salinity. The photobioreactors were continuously illuminated on the riser side at light intensity of 18 klx, and the airflow rate was kept at 0.5 L min^{-1}. At the end of the experiment, the entire biomass content was centrifuged (3000 × *g*, 10 min) and lyophilised.

2.3 Biomass Analysis

The carbon, hydrogen, nitrogen and sulfur contents of lyophilised biomasses were analysed in duplicate using an elemental analyser (ELEMENTAR Analysensysteme BmbH, model VARIOEL V5 19.9.23, Hanau, Germany). The lipid content was determined by the weight difference before and after extraction with petroleum ether (at 135 °C) using Soxhlet equipment (Foss/Soxtec 2055TM, Hillerød, Denmark). The carotenoids were extracted from the freeze-dried biomass and analysed by HPLC as described previously (Mercadante and Rodriguez-Amaya, 1998; Zanatta and Mercadante, 2007; Chagas *et al.*, 2015). For quantification, calibration curves were constructed for β-carotene (5 – 50 μg mL^{-1}), lutein (1 – 65 μg mL^{-1}) and zeaxanthin (1 – 40 μg mL^{-1}). The carotenoid standards were acquired from Sigma-Aldrich (Seelze, Germany). The limits of quantitation (LOQ) and detection (LOD) were respectively, 10.89×10^{-2} μg g^{-1} and 6.53×10^{-2} μg g^{-1} for β-carotene, 1.15×10^{-2} μg g^{-1} and 6.9×10^{-3} μg g^{-1} for lutein and 1.59×10^{-2} μg g^{-1} and 9.56×10^{-2} μg g^{-1} for zeaxanthin. Carotenoid quantification was performed by comparing the peak area of the sample with that of standards.

2.4 Statistical Analysis

Experimental data were analysed using *Statistica* 12.0 software (StatSoft, Inc. Tulsa, United States). Experimental results were approximated by a quadratic

polynomial equation (Equation 1):

$$Y = \beta_{\circ} + \beta_T x_T + \beta_S x_S + \beta_{T^2} x_T^2 + \beta_{S^2} x_S^2 + \beta_{TS} x_T x_S, \quad (1)$$

where x_T and x_S are regression variables (temperature and salinity, respectively), and Y represents the dependent variable, in these cases, lipid, β-carotene, lutein or zeaxanthin contents. The symbols β_0, β_T, β_S, β_T^2, β_S^2 and β_{TS} are regression coefficients of the model.

3 I RESULTS AND DISCUSSION

The carotenoid extract from *D. tertiolecta* showed six peaks as separated by HPLC (Figure 1 and Table 1). Peaks 3, 4 and 5 were all-*trans* forms of lutein, zeaxanthin and β-carotene, respectively. Peaks 1, 2 and 6 were identified based on the wavelengths of maximum absorption (λ_{max}) and spectral fine structural values (% III/II). The data agreed well with those of the literature for all-*trans*-neoxanthin, all-*trans*-antheraxanthin and 9-*cis*-β-carotene, respectively (De Rosso and Mercadante, 2007; Hu *et al.*, 2008).

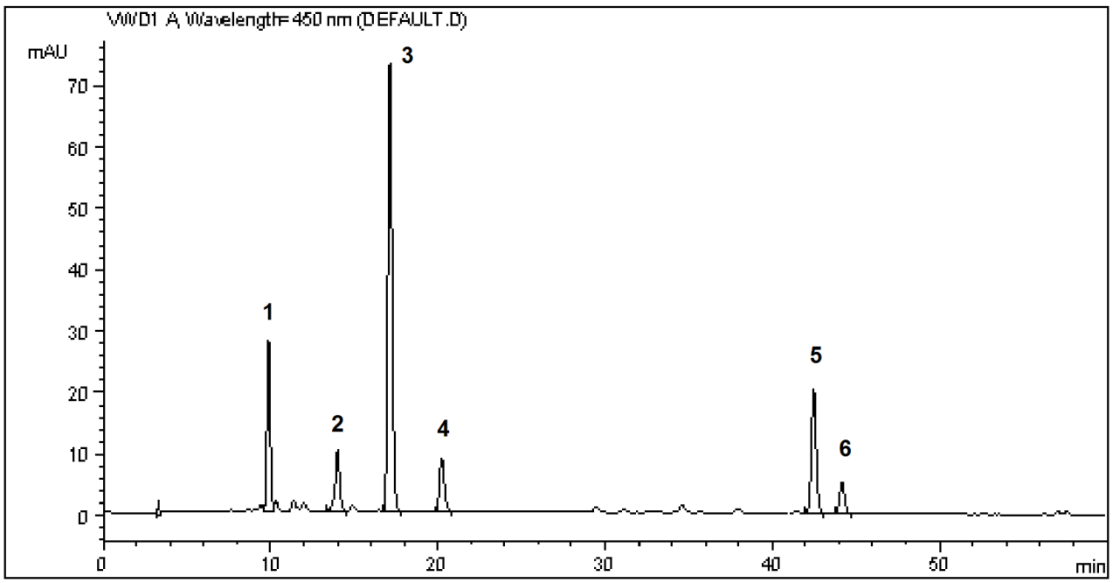

Figure 1: HPLC chromatogram of carotenoid extract from *Dunaliella tertiolecta*. Peak identification: (1) all-*trans*-neoxanthin, (2) all-*trans*-antheraxanthin, (3) all-*trans*-lutein, (4) all-*trans*-zeaxanthin, (5) all-*trans*-β-carotene, (6) 9-*cis*-β-carotene.

Peak number	Compound	Retention time (min)	λ_{max} (nm)			% III/II
1	all-*trans*-neoxanthin	9.9	415	438	468	85
2	all-*trans*-antheraxanthin	14.0	420	444	472	50
3	all-*trans*-lutein	17.1	418	443	471	60
4	all-trans-zeaxanthin	20.2	425	449	475	30
5	all-*trans*-β-carotene	42.5	421	451	476	20
6	9-*cis*-β-carotene	44.2	423	445	472	28

Table 1: Assignment data for carotenoids in Dunaliella tertiolecta.

λ_{max}: wavelengths of maximum absorption, % III/II: spectral fine structural value.

Members of the *Dunaliella* genera contain valuable carotenoid pigments such as α- and β-carotene, violaxanthin, neoxanthin, zeaxanthin and lutein (Tafreshi and Shariati, 2009). The absence of violaxanthin and the presence of the antheraxanthin and zeaxanthin in our *D. tertiolecta* extract can be explained by the xanthophyll cycle. Xanthophylls are essential for light-dependent growth. These molecules possesses various functions in photosynthetic organisms, mainly the protection against photo-oxidative damage. When light absorption exceeds photochemical utilisation, the xanthophyll cycle in higher plants and green algae consists of the conversion of violaxanthin via a de-epoxidation reaction to form antheraxanthin and subsequently, zeaxanthin resulting in the accumulation of zeaxanthin. In darkness or when light absorption is no longer in excess, antheroxanthin is converted to violaxanthin through reverse epoxidation. Thus, the xanthophyll cycle is a dynamically regulated reversible interconversion of violaxanthin to antheraxanthin to zeaxanthin; it occurs in the thylakoid membrane of photosynthetic cells and plays a role in photoprotection (Long *et al.*, 1994; Jin *et al.*, 2003). Formation of both antheraxanthin and zeaxanthin is closely associated with the use of continuous light in our experiments and the consequent irradiative stress in cells.

The carotenoids lutein and β-carotene were the major carotenoids accumulated under the assayed conditions. Under the test conditions, the highest amount of lutein, β-carotene and zeaxanthin attained in dry biomass were, respectively, 1315 ± 36 μg g^{-1}, 732 ± 51 μg g^{-1} and 244 ± 76 μg g^{-1}, at 93 h of culture.

The statistical analysis shows that both salinity and temperature affected the amounts of lutein, zeaxanthin and β-carotene produced by *D. tertiolecta* (Table 2). Response surfaces for lutein (Figure 2a) and β-carotene (Figure 2b) show that salinity had a strong effect on these carotenoids. The highest β-carotene content occurred between 0.7 and 0.8 M NaCl. The effect of temperature on the amount of β-carotene was negligible. The highest lutein content occurred at a salinity range slightly higher, between 0.70 and 0.85 M NaCl. The increase in the cultivation temperature slightly increased the lutein content. The response surface for zeaxanthin (Figure 2c) shows that salinity and temperature had a strong effect on the amount of zeaxanthin. The highest zeaxanthin content occurred between 0.7 and 0.8 M NaCl and between 26 °C

and 28 °C, and the maximum point occurred at 0.756 M NaCl and 26.8 °C.

	Lutein (μg g^{-1})		Zeaxanthin (μg g^{-1})		β-carotene (μg g^{-1})		Lipids (%)	
	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value	Coefficient	*p*-value
β_o	1310.554	0.0000	243.578	0.0000	631.860	0.0000	11.767	0.0000
β_T	-	-	-29.075	0.0336	-	-	0.8929	0.0040
β_{T^2}	-248.510	0.0172	-62.399	0.0089	-77.812	0.0881	-2.0071	0.0001
β_s	193.794	0.0013	35.275	0.0077	59.912	0.0474	-	-
β_{s^2}	-467.950	0.0001	-86.795	0.0006	-200.270	0.0002	-1.9194	0.0001
β_{Ts}	-	-	-	-	-	-	-	-
Regression								
p-value	< 0.0001		0.0011		0.0005		< 0.0001	
F	14.75		8.1		9.88		15.3	
R^2	0.735		0.682		0.622		0.754	
LOF[*]	0.386		0.8644		0.1044		0.6867	

Table 2: Values of the regression coefficients of the coded variables and regression parameters for the amount of lutein, zeaxanthin, β-carotene and total lipids at 93 h of cultivation of *Dunaliella tertiolecta*.[*] *p*-value of lack of fit

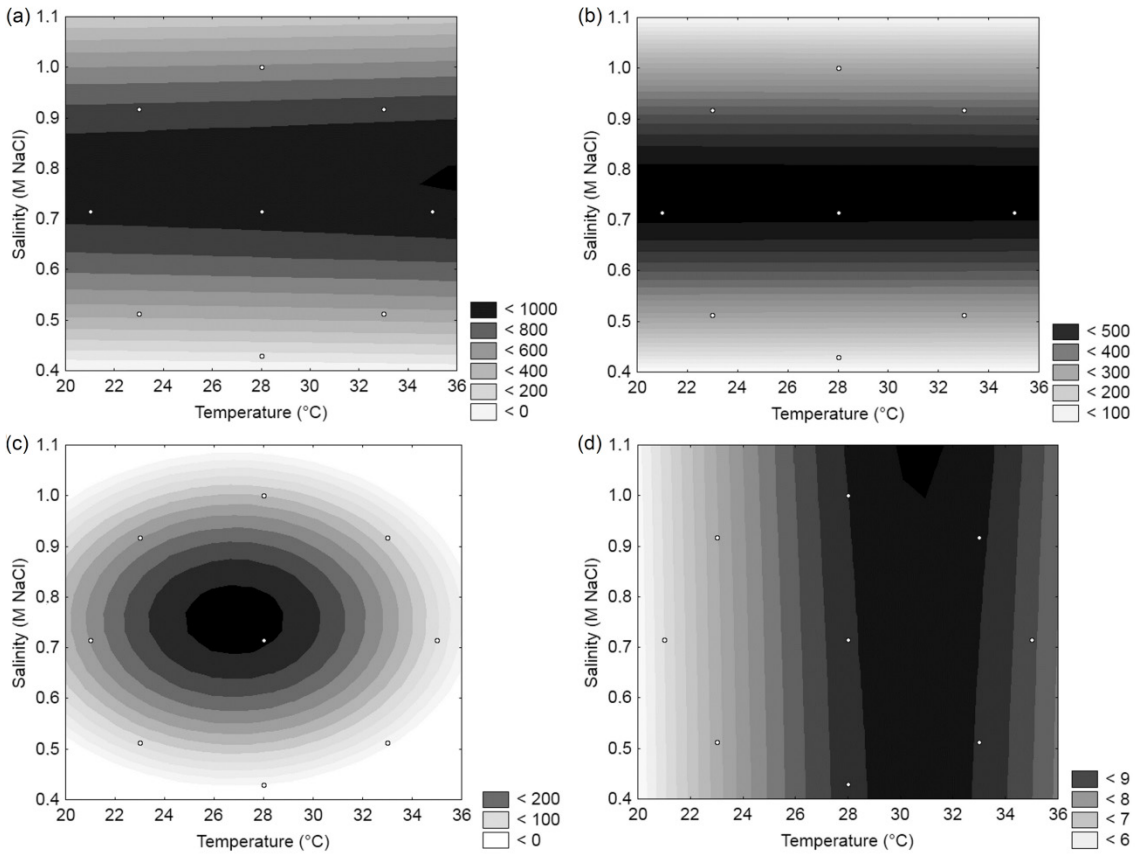

Figure 2**:** Response surfaces for the amounts of: (a) lutein (μg g^{-1}), (b) β-carotene (μg g^{-1}), (c) zeaxanthin (μg g^{-1}), (d) total lipids (%) at 93 h of culture as functions of the temperature and salinity.

There are studies that have investigated the production of β-carotene from *D. tertiolecta*, but few have examined the influence of physicochemical properties of the

medium on the production of lutein and zeaxanthin. Unlike the results of this work, *D. tertiolecta* DCCBC26 increased the total carotenoid content in the biomass when NaCl was increased from 0.5 M to 3 M (Fazeli *et al.*, 2006). Studies on *D. tertiolecta* CCAP 19/6B also showed that the lutein content in the biomass was about twice the β-carotene content (Barbosa *et al.*, 2005), similar to the results of this work.

Regarding the total lipid content in *D. tertiolecta*, temperature showed the strongest effect and the highest lipid content occurred at a temperature range between 28 °C and 32 °C. The increase in the medium salinity slightly increased the lipid content (Table 2, Figure 2d).

The highest experimental value of total lipid content (11.8 ± 1.8 %) was achieved in cultures performed at 28 °C and 0.715 M NaCl. In another work, *D. tertiolecta* SAG-13.86 presented a very similar lipid content (11.4 ± 1.8 %) (Sydney *et al.*, 2010). A review paper on the hydrothermal catalytic production of fuels and chemicals from aquatic biomass provides different lipid contents for different algae, ranging between 2 % and 22 % of dry matter, with a 6 % lipid content for *D. salina* (Becker, 2007).

Temperature and salinity showed no significant effect on *D. tertiolecta* elemental composition. Average values of carbon, hydrogen, nitrogen and sulfur content of all experiments were 41.19 ± 0.78 %, 3.48 ± 0.06 %, 1.55 ± 0.21 %, 0.36 ± 0.04 %, respectively. Shuping *et al.* (2010) found similar results for carbon (39 %), hydrogen (5.37 %) and nitrogen (1.99 %), while Sydney et al. (2010) found a carbon content of 36 %, both for *D. tertiolecta* biomasses.

4 I CONCLUSIONS

The experiments performed in this work showed that the physicochemical variables temperature and medium salinity strongly affected biomass composition during *D. tertiolecta* BE 003 growth. Temperature had a strong effect on the total lipid content, while medium salinity had a weaker effect on lipid content. However, medium salinity affected the concentration of the main carotenoids of *D. tertiolecta* biomass (lutein and β-carotene). Neither the temperature nor the salinity showed significant effect on *D. tertiolecta* elemental composition. These results show the importance of systematic studies on the effects of environmental factors on the microalgal growth parameters and biomass composition.

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