EVALUATION OF BIOMASS PRODUCTION, CAROTENOID LEVEL AND ANTIOXIDANT CAPACITY PRODUCED BY *THERMUS FILIFORMIS* USING FRACTIONAL FACTORIAL DESIGN

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ABSTRACT

A fractional factorial design 2^{5-1} was used to evaluate the effect of temperature, pH, and concentrations of yeast extract, tryptone and Nitsch's trace elements on the biomass, total carotenoids and protection against singlet oxygen by carotenoid extracts of the bacterium *Thermus filiformis*. In addition, the carotenoid composition was determined by high-performance liquid chromatography connected to a diode array and mass spectrometer detectors (HPLC-DAD-MS/MS). The production of biomass ranged from 0.113 to 0.658 g/L, the total carotenoid from 137.6 to 1,517.4 μg/g and the protection against singlet oxygen from 4.3 to 85.1 %. Results of the fractional factorial design showed that temperature had a negative effect on biomass production and a positive effect on carotenoid content and protection against singlet oxygen, besides, high levels of pH value, concentrations of yeast extract and tryptone had a positive effect on biomass production only at lower temperatures. The main carotenoids of *T. filiformis* were thermozeaxanthins. In the tested conditions, changes in the levels of the variables influenced the biomass, carotenoid production, and protection against singlet oxygen, although they did not influence the carotenoid profile. The results of this study provide a better understanding on the interactions among certain nutritional and cultivation conditions of a thermophile bacterium, *Thermus filiformis*, on biomass and carotenoid amounts, as well as on the antioxidant capacity.

Key words: thermophile bacterium, carotenoids, HPLC-DAD-MS/MS, antioxidant capacity, factorial design.

INTRODUCTION

Carotenoids belong to a group of natural pigments widely distributed in the nature with a huge diversity of chemical structures and functions. The main reason for using microorganisms to produce compounds that can otherwise be isolated from plants and animals or chemically synthesized is the ease of increasing production by environmental and genetic

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manipulation (19).

The interest in carotenoids has considerably increased in the last years due to the known evidence of their benefits to the human health (16, 24). One of the important characteristics of carotenoids is their ability to act as antioxidants, protecting cells and tissues from damaging effects of free radicals and singlet oxygen (O_2 ($^1\Delta_g$)). The free radicals and O_2 ($^1\Delta_g$) produced by the normal aerobic metabolism are highly reactive, and in case of oxidative stress, these oxidants can react with various components of living cells, such as proteins, DNA, or lipids, and cause structural changes leading to many diseases. The antioxidant mechanism of carotenoids is mainly attributed to their ability to quench O_2 ($^1\Delta_g$) and scavenge free radicals (26).

Several environments considered extreme and hostile to allow survival and growth of living organism are colonized by microorganisms that are specifically adapted to these niches. In this context, it is now recognized that extremophile microorganisms are a rich source of molecules of industrial interest and a great resource for developing new biotechnological applications that can revolutionize our daily lives and the advancement of knowledge (25). One of the properties of such microorganisms is their ability to adapt to extreme environments, in which factors, such as pH, temperature, pressure and salt concentration, exceed the values considered as standard for most living beings (11).

Among all extreme environments, the temperature is the parameter that most influence on the function of biomolecules and the maintenance of the biological structures. High temperature can lead to denaturation of the enzymes responsible by carotenoids biosynthesis (1); however, extremophile enzymes are characterized by higher thermo stability and reaction rate, with optimum activity as high as 90°C (21). The existence of stable geothermal environments has allowed the selection or the persistence of microorganisms that not only survive but also need high temperatures to survive (11).

Bacteria of the genus *Thermus*, belong to the Thermaceae family, are Gram negative, aerobic and catalase positive. In general, *Thermus* bacteria have an optimum growth between 70 and 75°C; however, some species grow at lower temperatures, around 60°C. The optimum pH range for these bacteria is between 7.5 and 8.0, although they can grow at lower (~ 5.1) or higher (~ 9.5) pH values (13). According to Brock and Freeze (5), the bacteria from *Thermus* genus can use amino acids or ammonium salts as nitrogen sources and sugar or organic acids as carbon sources. Unlike typical Gram-negative bacteria, the outer membranes of *Thermus* species are not composed of lipopolysaccharides but of peculiar glycolipids, which structures seem to be strictly involved in their adaptation to high temperatures (17).

The bacterium *Thermus filiformis* was first isolated in 1987 from a hot spring in New Zealand. This bacterium synthesizes yellow carotenoids, low levels of iso-branched fatty acids, and high levels of anteiso-branched fatty acids (14). These characteristics distinguish *T. filiformis* from all other *Thermus* species.

Nutritional and physical factors, such as concentration and type of nitrogen and carbon sources, minerals, pH, aeration, temperature and light have an influence on the cellular growth, composition and production of carotenoids by microorganisms (3). Therefore, studies to improve conditions and composition of the culture medium should be carried out for each microorganism. Even though, most studies were conducted with fungi, yeast and microalgae, mainly related to the production of astaxanthin by *Haematococcus pluvialis* (23) and *Phaffia rhodozyma* (10), and to the production of β-carotene by *Dunaliella* sp. (32) and *Blakeslea trispora* (9).

The aims of this study were to determine the simultaneous influence of temperature, pH, concentrations of yeast extract, tryptone and Nitsch's trace elements on the biomass and carotenoid production, as well as on the protection against O_2 ($^1\Delta_g$) by the carotenoid extracts produced by *T. filiformis* ATCC 43280, using a fractional factorial design and evaluate

the carotenoid profile by high-performance liquid chromatography connected to a diode array and mass spectrometer detectors (HPLC-DAD-MS/MS). *T. filiformis* was chosen because, although it has been firstly described in 1987, only genetic studies were found in the literature (6, 15). In fact, to the best of our knowledge, no report is available in the literature regarding the use of fractional factorial design to study the biomass, carotenoid production or protection against singlet oxygen of any bacteria from the *Thermus* genus.

MATERIALS AND METHODS

Bacterial strain

The strain of the bacterium *Thermus filiformis (*ATCC 43280) was obtained from the "Laboratório de Materiais de Referência do Instituto Nacional de Controle de Qualidade em Saúde da Fundação Oswaldo Cruz", located in Rio de Janeiro, Brazil. The strain was maintained in the Castenholz TYE 1% medium and a stock culture was stored at -70°C in Castenholz TYE 1% broth with glycerol.

Culture medium and cultivation conditions

Castenholz TYE 1 % medium was sterilized in autoclave at 120°C for 20 min at 1 atm (14). The culture medium was changed according to the different conditions of the experimental design shown in Tables 1 and 2.

Table 1. Independent variable levels used in the fractional factorial design

Independent Variable		Level (coded)		
		- 1	0	+ 1
X_1	Temperature (°C)	65	70	75
X_2	pH	7.0	7.5	8.0
X_3	Tryptone (g/L)	5.0	7.5	10.0
X_4	Yeast extract (g/L)	5.0	7.5	10.0
X_5	Nitsch's trace elements (mL/L)	2.0	3.0	4.0

The cultivation of the bacterium was carried out in three consecutive phases. In the first phase, the bacterium was grown in Castenholz TYE 1 % medium and incubated at 70°C for 24 hours. For the second phase (pre-inoculum), part of the culture was transferred to Erlenmeyer flasks of 500 mL, containing culture broth, following by incubation at 70°C, 230 rpm during 48 hours. The pre-inoculum reached a cellular density of 10^9 cells/mL.

In the third phase, the inoculum was prepared in Erlenmeyer flasks of 1,000 mL, with 10% (v/v) of pre-inoculum, followed by incubation at the same conditions used in the pre-inoculum.

Experimental design and statistical analysis

A fractional factorial design was used in order to evaluate the effect of certain conditions of the culture medium and

cultivation on dry biomass production (g/L of culture broth), total carotenoids (µg/g of dry biomass) and protection against O_2 ($^1\Delta_g$) by the carotenoid extract (%). The values for the levels of each independent variable: temperature (°C), pH, concentrations of yeast extract (g/L), tryptone (g/L) and Nitsch's trace elements (mL/L), Table 1, were established based on literature data about thermophile microorganisms (2, 14). A fractional factorial design 2^{5-1} with 3 central points was used, totalizing 19 experiments (Table 2).

The experimental responses were analyzed using the "Experimental Design" module of the Statistica 6.0 program (29) to determine the effect of the independent variables (temperature, pH, concentrations of yeast extract, tryptone and Nitsch's trace elements) and the interaction among them on the following responses: biomass production, total carotenoid levels and protection against O_2 ($^1\Delta_g$).

Table 2. Matrix with coded values of the fractional factorial design 2^{5-1} and responses obtained for *Thermus filiformis*

	Variable level[a]					Response		
Assay	X_1	X_2	X_3	X_4	X_5	Biomass (g/L)	Carotenoids (µg/g)	% protection[b]
1	-1 (65)	-1 (7.0)	-1 (5.0)	-1 (5.0)	1 (4.0)	0.380	475.1	51.5
2	1 (75)	-1 (7.0)	-1 (5.0)	-1 (5.0)	-1 (2.0)	0.380	1,517.4	85.1
3	-1 (65)	1 (8.0)	-1 (5.0)	-1 (5.0)	-1 (2.0)	0.329	383.9	46.1
4	1 (75)	1 (8.0)	-1 (5.0)	-1 (5.0)	1 (4.0)	0.380	823.6	49.0
5	-1 (65)	-1 (7.0)	1 (10.0)	-1 (5.0)	-1 (2.0)	0.476	473.5	33.6
6	1 (75)	-1 (7.0)	1 (10.0)	-1 (5.0)	1 (4.0)	0.451	1,093.9	82.9
7	-1 (65)	1 (8.0)	1 (10.0)	-1 (5.0)	1 (4.0)	0.464	489.6	57.1
8	1 (75)	1 (8.0)	1 (10.0)	-1 (5.0)	-1 (2.0)	0.384	1,328.6	71.9
9	-1 (65)	-1 (7.0)	-1 (5.0)	1 (10.0)	-1 (2.0)	0.491	688.2	34.4
10	1 (75)	-1 (7.0)	-1 (5.0)	1 (10.0)	1 (4.0)	0.520	1,019.6	42.7
11	-1(65)	1 (8.0)	-1 (5.0)	1 (10.0)	1 (4.0)	0.504	493.0	31.4
12	1 (75)	1 (8.0)	-1(5.0)	1 (10.0)	-1 (2.0)	0.113	559.4	64.8
13	-1 (65)	-1 (7.0)	1 (10.0)	1 (10.0)	1 (4.0)	0.376	137.6	4.3
14	1 (75)	-1 (7.0)	1 (10.0)	1 (10.0)	-1 (2.0)	0.169	845.6	40.4
15	-1 (65)	1 (8.0)	1 (10.0)	1 (10.0)	-1 (2.0)	0.658	336.5	48.9
16	1 (75)	1 (8.0)	1 (10.0)	1 (10.0)	1 (4.0)	0.122	694.9	60.5
17	0 (70)	0 (7.5)	0 (7.5)	0 (7.5)	0 (3.0)	0.327	318.9	65.7
18	0 (70)	0 (7.5)	0 (7.5)	0 (7.5)	0 (3.0)	0.322	369.9	42.2
19	0 (70)	0 (7.5)	0 (7.5)	0 (7.5)	0 (3.0)	0.331	276.7	39.0

X_1 – temperature (°C); X_2 – pH; X_3 – yeast extract (g/L); X_4 – tryptone (g/L); X_5 - Nitsch's trace elements (mL/L)
[a]real values in brackets
[b]% of DMA protection against O_2 ($^1\Delta_g$)

Determination of dry biomass

The inoculum was centrifuged at 23,900x*g* by 10 min at 10°C in refrigerated centrifuge (Sorval Instruments RC5C – Dupont®) and the supernatant was discarded. The cellular mass was washed once with distilled water followed by centrifugation. The pellet was frozen in Petri dishes at -35°C for later freeze-drying and weighing for quantification of the dry biomass produced by culture liter.

Determination of carotenoids

The carotenoid extraction was carried out according to the method of Squina and Mercadante (28), with some modifications. The carotenoids were exhaustively extracted by maceration of dry biomass (30 mg) with ethyl acetate followed by methanol in porcelain capsules with glass beads and centrifuged at 1,100x*g* for 5 min. This procedure was repeated several times until the biomass became colorless. The carotenoid extract was filtered in polyethylene membrane with 0.22 µm pore, dried under nitrogen flow and stored in the freezer (T < -35°C) under nitrogen atmosphere.

The total carotenoid content was calculated using the absorbance measured at the maximum wavelength (λ_{max}) in a diode array spectrophotometer (Agilent model 8453, Palo Alto, USA) in the range from 220 to 700 nm. The concentration of carotenoids was calculated using the Lambert - Beer law and absorption coefficient of 2450 for zeaxanthin in ethanol (4).

In addition, the carotenoids were analyzed by HPLC-DAD-MS/MS. The carotenoids were separated on a C_{30} YMC column (5 µm, 4.6 x 250 mm) with a linear gradient of methanol/methyl *tert*-butyl ether from 95:5 to 70:30 in 30 min, followed by 50:50 in 10 min at 0.9 mL/min and column temperature set at 32ºC (7). The carotenoids were identified considering the elution order on the C_{30} YMC column, characteristics of the UV-visible spectra, protonated molecule ([M+H]$^+$) and MS/MS fragments (data not shown).

Determination of antioxidant capacity

The percentage of protection against O_2 ($^1\Delta_g$) was

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performed according to Montenegro et al. (20), with some modifications. The 9,10-dimethylanthracene (DMA) in ethyl acetate was used as actinometer and methylene blue in ethanol as sensitizer. The system was submitted to an excitation source with a 150 W filament xenon lamp coupled to a set of red and orange cut-off filter to avoid direct excitation of the carotenoids. The reaction was monitored by absorbance measurements between 200 and 800 nm during 10 min.

The protection was calculated according to equation 1.

$$\% \ protection = \frac{k_{obs}^{DMA} - k_{obs}^{DMA+CAR}}{k_{obs}^{DMA}} \times 100 \qquad (1)$$

where k_{obs}^{DMA} and $k_{obs}^{DMA+CAR}$ were the first order rate constants for DMA (absorbance at 374 nm) in the absence and presence of carotenoid extract, respectively.

RESULTS

The results summarized in Table 2 show the influence of temperature, pH, concentrations of yeast extract, tryptone and Nitsch's trace elements on the contents of dry biomass, total carotenoid and protection against O_2 ($^1\Delta_g$) by carotenoid extracts, using a fractional factorial design. The effect of each factor and their interactions were obtained with confidence interval of 95 % (Table 3).

Table 3. Effect of the variables on the biomass production, total carotenoid production and protection against singlet oxygen (only the significant values are shown).

Response	Effect of the Significant Variables		p	R^2
Biomass production (g/L)	X_1	-0.15	< 0.001	
	X_1*X_2	-0.09	0.002	
	X_1*X_3	-0.07	0.015	
	X_1*X_4	-0.13	< 0.001	0.96
	X_1*X_5	0.08	0.005	
	X_2*X_3	0.08	0.008	
	X_3*X_4	-0.08	0.008	
	X_3*X_5	-0.09	0.003	
Total carotenoid production (µg/g)	X_1	550.72	0.001	0.82
	X_4	-226.34	0.090	
% of protection	X_1	3.96	< 0.001	
	X_4	-3.12	0.001	0.91
	X_2*X_3	2.08	0.014	
	X_2*X_4	2.35	0.008	

X_1 – temperature; X_2 – pH; X_3 – yeast extract; X_4 – tryptone; X_5 - Nitsch's trace elements.

Effect of the independent variables on biomass production

The assay with the highest yield of biomass was number 15 (Table 2) with 0.658 g/L where the variables temperature and concentration of Nitsch's trace elements were adjusted at the lowest level (65°C and 2.0 mL/L, respectively) and pH, concentrations of yeast extract and tryptone at the highest level (8.0, 10.0 and 10.0 g/L, respectively). The lowest biomass production was observed in assay number 12 with 0.113 g/L

where the values of temperature, pH and concentration of tryptone were adjusted at 75°C, 8.0 and 10.0 g/L, respectively, whilst concentrations of yeast extract and Nitsch's trace elements at 10.0 g/L and 2.0 mL/L, respectively.

The results reported in Table 3 show that the model for biomass production has a R-square of 0.96. The R-square value is an indicator of how well the model fits the data, i.e., an R-square close to 1.0 indicates that almost all of the variability

was accounted with the variables specified in the model. For biomass production, only temperature had a significant linear effect (p < 0.001), with higher biomass production at lower temperatures. In addition, interactions of temperature with pH, concentrations of yeast extract, tryptone and Nitsch's trace elements also had a significant effect on biomass production.

Effect of the independent variables on carotenoid production

The highest amount of total carotenoids (1,517.4 μg/g) was observed in assay number 2, where the independent variables were adjusted at the lowest level with exception of the temperature, which was set at the highest level. On the other hand, the lowest carotenoid production (137.6 μg/g) was observed in assay number 13 where only temperature and pH were set at the lowest level.

The model adjusted for total carotenoid production had a R-square of 0.82, and both temperature and concentration of tryptone had a significant linear effect (p<0.001 and p<0.090, respectively). Temperature had a positive effect (550.72) whilst concentration of tryptone had a negative effect (-226.34). No interactions among the independent variables had significant effect on carotenoid production.

In order to verify the composition of carotenoids produced by *T. filiformis*, some assays were analyzed by HPLC-DAD-MS/MS. Independently of the cultivation conditions the identified carotenoids were the same in all the samples. Seven carotenoids produced by the strain *T. filiformis* were identified: zeaxanthin monoglucoside, all-*trans*-zeaxanthin and zeaxanthin monoglucoside esterified with branched fatty acids containing 11, 12, 13, 14 and 15 carbons (Figure 1). In all the assays, the major carotenoid was thermozeaxanthin-15 showing percentage ranging from 34.0 to 59.2 % (Figure 2, Table 4).
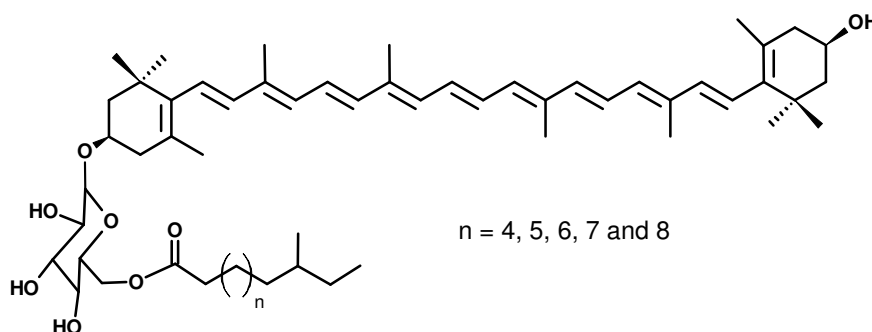

n = 4, 5, 6, 7 and 8

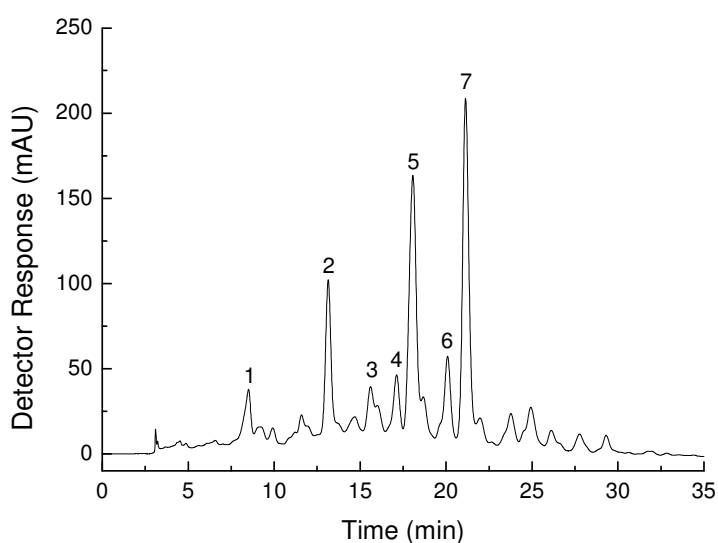**Figure 1.** Zeaxanthin monoglucoside acylated with branched fatty acids

Figure 2. Chromatogram, obtained by HPLC-DAD, of the carotenoid extract from *Thermus filiformis* obtained in assay 8. Chromatographic conditions are described in the text. Detection was at 450 nm. Peak identification: 1. zeaxanthin monoglucoside; 2. all-*trans*-zeaxanthin; 3. thermozeaxanthin-11; 4. thermozeaxanthin-12; 5. thermozeaxanthin-13; 6. thermozeaxanthin-14; 7. thermozeaxanthin-15.

According to the results presented in table 4, the range of temperature studied showed influence on the carotenoid amounts, although it did not favor the biosynthesis of any particular carotenoid. On the other hand, lower pH value (pH 7.0) seems to favor the production of thermozeaxanthin–15 (59.2 %) over thermozeaxanthin–13 (19.6 %) as verified for assay 2, since at pH values of 7.5 (assay 17) and 8.0 (assays 8

and 15) the percentages of thermozeaxanthin–13 (30.0 to 32.8 %) and thermozeaxanthin–15 (34.0 to 36.6 %) were similar. In addition, all-*trans*-zeaxanthin was produced in higher amounts when the pH of the medium was adjusted at higher values, indicating that the expression of *crtX*, the gene coding for zeaxanthin glucosylase, seems to be inhibited at higher pH values.

Table 4. Main carotenoids produced by *T. filiformis*

Peak	Carotenoid	Area (%)*			
		Assay 2[†]	Assay 8[†]	Assay 15[†]	Assay 17[†]
1	zeaxanthin monoglucoside	4.5	4.3	6.9	5.3
2	all-*trans*-zeaxanthin	4.4	13.2	9.7	8.2
3	thermozeaxanthin-11	4.1	5.9	7.7	6.6
4	thermozeaxanthin-12	3.3	4.8	3.1	4.1
5	thermozeaxanthin-13	19.6	30.0	32.6	32.8
6	thermozeaxanthin-14	4.2	7.9	4.6	6.3
7	thermozeaxanthin-15	59.2	34.0	35.4	36.6

* percentage of area from the chromatogram peaks
[†] the culture conditions of these assays are shown on Tables 1 and 2.

Effect of the independent variables on antioxidant capacity

Since the carotenoid profiles were similar in different conditions, the protection against $O_2(^1\Delta_g)$ by carotenoid extracts showed a behavior that is only dependent on the amount of carotenoids present in each extract. Therefore, the highest percentage of protection against singlet oxygen (85.1 %) was observed in the assay with the highest carotenoid content (assay 2) and the lowest percentage of protection against singlet oxygen (4.3 %) was found in the assay with the lowest content of carotenoid (assay 13).

The model developed for antioxidant activity had a R-square of 0.91. As well as for total carotenoid production, temperature and concentration of tryptone also had significant linear effect (p<0.001 and p<0.014, respectively). Moreover, the interaction between pH and concentration of yeast extract and pH and concentration of tryptone were also significant for the antioxidant activity.

DISCUSSION

In the present study, the highest biomass amount (0.658 g/L) was produced in the medium with the highest concentrations of yeast extract and tryptone (10 g/L). In disagreement with this data, Brock and Freeze (5) observed an inhibitory effect of carbon and nitrogen sources on the cell growth cultures of *T. aquaticus* with high tryptone and yeast extract concentrations (10 g/L) at pH 7.1 (quantitative values were not reported). On the other hand, Saiki et al. (27) isolated a *Thermus* strain that grew in a medium with 1% tryptone and yeast extract indicating that the problem with rich culture medium was a decrease in the pH value at high temperature. This fact can be confirmed by the results observed in assays 13 and 15 (Table 2), since both were performed at the same temperature and concentrations of yeast extract and tryptone but at different pH values, because at pH 7.0 half of the

biomass amount was produced (0.376 g/L) as compared to the assay with pH 8.0 (0.658 g/L).

A positive influence of high temperatures (≈ 75°C) on carotenoid production was found in the present study, rising more than 10 times when assays 2 and 13 are compared. According to the literature, other microorganisms also had an increase in carotenoid production at higher temperatures. Tjahjono et al. (31) suggested that the growth of *Haematococcus pluvialis* at high temperatures (30°C) might enhance the formation of active oxygen species in the cells, which is responsible for the stimulation of carotenogenesis. A threefold increase in the carotenoid content was observed when the temperature of *Mucor rouxii* cultures was raised to 37°C (165 µg of carotenoids/g dry weight) as compared to the amount obtained at the optimum growth temperature of 28 °C (55 µg of carotenoids/g dry weight) (22).

The biomass and carotenoid production have an inverse behavior in relation to temperature; while lower temperatures had a positive effect on biomass production higher temperature were preferable for carotenoid production. A similar behavior was also noticed in investigations about lipolytic enzyme production by *Thermus thermophilus* (8). The others variables (pH, concentrations of yeast extract, tryptone and Nitsch's trace elements) showed no comparable behavior in relation to both biomass and carotenoid production.

The carotenoids identified in *T. filiformis* were mainly thermozeaxantins, which are formed by two β-ionone rings plus a glucose moiety acylated by a branched fatty acid. This type of carotenoids is probably associated with the membrane stabilization of these microorganisms by inserting its hydrophobic core into the lipid bilayer (12). Moreover, membrane stabilization is considered essential for the growth of thermophile bacteria at high temperatures and bacterial carotenoids play an important role in reinforcing the membrane (18).

As expected, the protection against singlet oxygen and total carotenoid production showed a direct relationship, the assays with higher amounts of carotenoids had greater percentage of DMA protection. The O_2 ($^1\Delta_g$) quenching activity of carotenoids in organic solvent is mainly dependent on the number of conjugated double bonds and to a lesser extent influenced by carotenoid end groups (cyclic or acyclic) or the nature of substituents in the cyclic end groups (30). It is also well known that carotenoids are efficient quenchers of both excited triplet state of photosensitizers and singlet oxygen, mainly by an energy-transfer mechanism (20).

In conclusion, evaluation of the influence of temperature, pH, concentrations of yeast extract, tryptone and Nitsch's trace elements allowed the definition of favorable conditions for biomass, carotenoid production and percentage of protection against singlet oxygen for *T. filiformis*. Since the effect of temperature was significant but contrary for biomass amount and production of carotenoids, the use of a two-phase cultivation process, where the production of carotenoids is separated from the cellular growth stage, would allow the achievement of both, high levels of cellular growth and of carotenoids.

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