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Growth of the microalgae *Tetraselmis tetrathele* and nitrate depletion in culture medium Guillard f/2 and Conway

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ABSTRACT. This study aimed to evaluate the effect of nutrient depletion during the cultivation of the microalgae *Tetraselmis tetrathele*, and to verify the algal growth and productivity in different culture media (Guillard f/2 and Conway). The experiment was conducted in nine-liter containers, with working volume of eight liters, salinity 35 ± 2‰, temperature 28 ± 1°C, with constant light and aeration, around 200 μE cm^{-2} s^{-1} and 3 L air $min.^{-1}$. Results showed that microalgae development caused nutrient depletion, since we observed an inversely proportional relationship between nitrate concentration and algal biomass during the cultivation. Regarding the culture media, a higher algal productivity was observed in the cultivation developed in Conway medium ($p < 0.05$), obtaining at the end of the cultivation, biomass of 3.38 ± 0.02 g L^{-1}. In the Guillard medium, only 2.47 ± 0.05 g L^{-1} was obtained. The cultivation in Conway medium presented better results concerning microalgae growth, with a more pronounced exponential phase. This was due to the higher nitrate availability, which was reduced more rapidly in the culture medium Guillard f/2.

Keywords: algal concentration, yield, nutrients.

Crescimento da microalga *Tetraselmis tetrathele* e depleção de nitrato em meio de cultivo Guillard f/2 e Conway

RESUMO. O presente trabalho teve como objetivo avaliar o efeito da depleção de nutrientes durante o cultivo da microalga *Tetraselmis tetrathele*, bem como verificar o crescimento e a produtividade algal em diferentes meios de cultivo (Guillard f/2 e Conway). O experimento foi realizado em recipientes de nove litros, com volume útil de oito litros, salinidade de 35 ± 2‰, temperatura de 28 ± 1°C, com iluminação e aeração constantes, em torno de 200 μE cm^{-2} s^{-1} e 3 L ar $min.^{-1}$. Os resultados mostraram que o desenvolvimento da microalga resultou na depleção de nutrientes, uma vez que foi observada uma relação inversamente proporcional entre a concentração de nitrato e a biomassa algal durante o cultivo. Em relação aos meios de cultivo, foi observada maior produtividade algal na cultura desenvolvida em meio Conway ($p < 0,05$), obtendo, ao final do cultivo, biomassa de 3,38 ± 0,02 g L^{-1}. No meio Guillard foi obtido apenas 2,47 ± 0,05 g L^{-1}. O cultivo em meio Conway apresentou resultados melhores quanto ao crescimento microalgal, dispondo de uma fase exponencial mais acentuada. Isto por conta da maior disponibilidade do nitrato, o qual foi reduzido mais rapidamente no cultivo em meio Guillard f/2.

Palavras-chave: concentração, produtividade, nutrientes.

Introduction

Microalgae are unicellular organisms able to use solar energy and carbon dioxide with photosynthetic efficiency higher than the plants for the production of biomass. Growth stage in microalgae cultures and the manipulation of the physical and chemical conditions of the cultures may result in differences in cell composition, i.e., variations in levels of lipid, protein, carbohydrate and other components of the cell (LOURENÇO, 2006). This trait coupled to culture techniques makes the microalgae one of the priority subjects for the most modern research areas. This is due to its key role in several trophic chains and in the possibility of commercial application in distinct areas as nutrition, human and animal health, wastewater treatment, energy production, and obtaining compounds of interest for food, chemical and pharmaceutical industries (GROBBELAAR, 2004).

According to Pulz and Gross (2004), the market of functional food, using microalgae in pasta, bread, yogurt and beverage, has fast development in countries like France, United States, China and Thailand. The most common application is in aquaculture, as direct or indirect food for some species of fish and mollusk and other animals (DERNER et al., 2006). Studies have proved that

microalgae are a potential source of biodiesel (KALIN et al., 2005; MALLICK, 2002; MUNOZ; GUIEYSSE, 2006; SURESH; RAVISHANKAR, 2004) and may become an alternative to fossil fuels in the future (GAVRILESCU; CHIST, 2005).

Lourenço (2006) reports that the interaction of growing microalgae with the culture medium and with their own physical environment results in expressive changes in cell density, that are prone to increase numerically at great proportions after inoculation. Nevertheless, in contrast, the concentration of dissolved nutrients tends to drastically reduce over the time, reaching complete depletion, depending on the time of culture development.

Several studies have shown the effect of different culture media for production of specific compounds in various areas, such as pharmaceutical production of hydrocarbons and exopolysaccharides in autotrophic cultures (DAYANANDAA et al., 2007), and about the influence of low-cost culture media on the chemical composition of cultivation medium (RAOOF et al., 2006; SASSANO et al., 2010).

Nutrient variation can cause increase or reduction in compounds present in the algae (SANTOS et al., 2003). Chen et al. (2010) observed similarities in fatty acid composition from *Dunaliella tertiolecta* cultivated in normal growth conditions in comparison to nutrient deprivation. The filamentous algae *Schizomeris leibleinii* achieved rapid growth under low nitrate concentration, but had better results at high concentrations of this nutrient (PEREIRA; BRANCO, 2006).

This study aimed at assessing the growth and effect of nitrate depletion in cultures of the microalgae *T. tetrathele*, using two different culture media (Guillard f/2 and Conway).

Material and methods

Microalgae *T. tetrathele* was obtained from strain database of the Laboratory of the Center for Aquaculture Technology of the Department of Fishing Engineering from the Federal University of Ceará. The culture media used for the maintenance of inoculum and accomplishment of the experiments were: Guillard f/2 (GUILLARD, 1975) and Conway (WALNE, 1966), whose composition can be observed in Tables 1 and 2, respectively. A sample of a pre-established culture of *T. tetrathele* was diluted to determine the optical density. To determine biomass (g L^{-1}), each sample was centrifuged (12.000 x g; 5 min.), washed twice with distilled water and centrifuged again. Afterwards, the samples were filtered; oven dried at 105°C for 16h and weighed on analytical balance (TAKAGI et al., 2006). Finally, it was established the correlation between the optic density (DO_{680}) and the dry weight (g L^{-1}) to determine the linear regression equation between the variables.

Tabe 1. Composition of modified Conway culture medium.

Solution	Reagent	Stock solution (mg 1.000 mL^{-1})	Culture médium (mL)
1	Na_2EDTA	45.0	
	$NaNO_3$	100.0	
	H_3BO_3	33.6	
	Na_2HPO_4	20.0	1.0
	$MnCl_2.4H_2O$	0.36	
	$FeCl_3.6H_2O$	1.3	
2	$ZnCl_2$	2.1	
	$CaCl_2$	2.0	
	$(NH_4)_6\ Mo_7O_{24}.4H_2O$	0.9	0.1
	$CuSO_4.5H_2O$	2.0	
		100	
3	Vitamin B12	10	0.1
	Vitamin B1	200	0.1
3	Distilled water	-	1.000

Table 2. Composition of Guillard f/2 culture medium.

Solution	Reagent	Stock solution (g 1.000 mL^{-1})	Culture medium (mL)
1			
3	$NaNO_3$	75.0	1.0
4			
2	$NaH_2\ PO_4\ H_2O$	5.0	1.0
	$Na_2O(SiO_2)2$	12.0	
3	$CuSO_4$	9.8	1.0
	$ZnSO_4$	22.0	
	$MgCl_2$	10.0	
	$Na_2MoO_4.2H_2O$	6.3	
4	$FeCl_2$	3.0	1.0
	Na_2 EDTA 2H2O	4.6	
	Thiamine hydrochloride	2.0	
5	Pyridoxine hydrochloride	2.0	0.5
	Cyanocobalamin	0.1	
6	Distilled water		1.000

The experiment was conducted in quadruplicate, using nine-liter containers, with working volume of eight liters, five liters of each culture medium, and three liters of a pre-culture (*inoculums*), thus characterizing a stationary cultivation, in which, after inoculation there is no addition of fresh culture during the culture development (LOURENÇO, 2006). The culture media and the containers were previously sterilized in an autoclave for 20 minutes at 122°C to prevent culture contamination.

The cultivations were submitted to constant aeration through diaphragm pump with air flow of 3 L $min.^{-1}$, salinity of 35 ± 2‰. Room temperature and light intensity were kept at 28 ± 1°C and 200 µE cm^{-2} s^{-1}, using 40W fluorescent lamps, and the experiment was performed under continuous light.

Cell concentration was determined daily through the regression equation between OD_{680} and biomass (g L^{-1}), according to Xu et al., (2006).The growth rates (K) in divisions per day and the maximum cell concentration (g L^{-1}), in the treatments, were

obtained at the day with higher yield of the cultures (OHSE et al., 2008), being the K calculated as described by Lourenço (2006).

After the cultivation, microalgae were flocculated with a strong base (2.0 N NaOH 2N), the flakes were washed three times with deionized water in order to zero salinity, oven dried at 60°C for 24h, and weighed on analytical balance.

Nitrate determination was carried out every 48h in 25 mL-samples that were centrifuged at 12.000 x g for 5 min. at 28ºC to separate algal cells. Initially, a centrifuged sample was used as a blank, which was taken to the spectrophotometer at a wavelength 500 nm, in order to zero the reading device. In other sample, we added nitraVer 5 Nitrate reagent and after the reaction time of 5 min., the same was taken to the spectrophotometer and the reading expressed in mg L^{-1}.

To test whether there was statistical difference among the mean values of the growth parameters in the two culture media, we performed a Student's t-test. The mean values of the nitrate concentrations in the different phases of the growth curve were submitted to an analysis of variance (ANOVA), and in the case of significant difference, we applied the Tukey's test to compare the means. All analyses were performed at 5% statistical significance level.

Results and discussion

Microalgae concentration of the cultivations was determined by spectrophotometry through the OD_{680nm} and converted to biomass (g L^{-1}) with the regression equation (Figure 1). We observed a strong positive correlation (R^2 = 0.99) between the variables, since when the OD_{680nm} has raised, also increased the biomass (g L^{-1}).

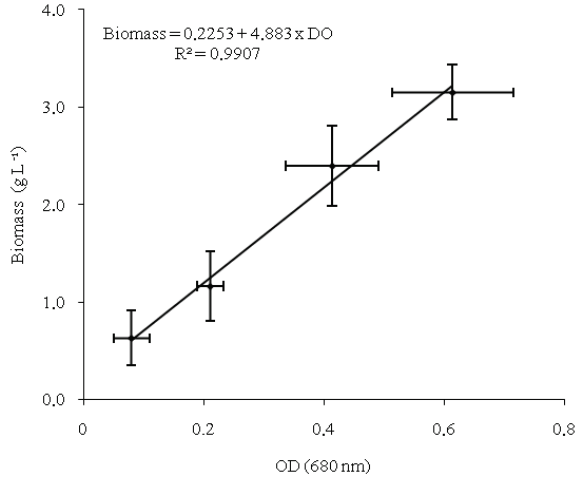

Figure 1. Linear correlation between OD680nm and biomass (g L-1), linear regression line and coefficient of determination (R2). Each dot is the average of four replicates ± standard deviation.

The daily mean growth rate in Guillard f/2 culture medium was 0.412 ± 0.06 divisions day^{-1}, statistically similar (α=0.05) to obtained for Conway culture medium (Table 3). This similarity probably occurred due to the same culture conditions provided and same initial concentrations of nutrients in both culture media, since we also did not observe statistical difference ($p < 0.05$) in the initial concentration of nitrate in both treatments.

According to Tonon et al. (2002), nutrient depletion in microalgae cultivations can be monitored through nitrate determination. In this way, over the time, it was verified a decrease in nitrate concentration whereas biomass increased, due to the assimilation of this compound by the microalgae (*Nannochloropsis oculata, Phaeodactylum tricornutum, Thalassiosira pseudonana*) in the medium (LOURENÇO, 2006). Guzmán-Murillo et al. (2007), analyzing different culture media to the microalgae *Phaeodactylum tricornutum*, evidenced that cell density was not affected by different nitrogen concentrations, with high cell density both in test with high and low concentration of nitrogen.

Zittelli et al. (2006), examined the productivity and photosynthetic efficiency of the microalgae T. suecica and achieved growth rates of up to 2.13 ± 0.35 division day^{-1}. This relatively high growth rate occurred due to high luminosity, since the cultures were performed externally in tubular photobioreactor, subjected to solar radiation. Ohse et al. (2008), investigating the cell growth of several microalgae in autotrophic system, observed that the species *T. chuii* and *T. suecica* also presented growth rates of 1.28 ± 0.12 and 1.54 ± 0.08 divisions day^{-1}, respectively. Meseck et al. (2005) registered the effect of different light intensities and photoperiods, achieving growth rates of up to 0.61 ± 0.04 divisions day^{-1}, similar to found in the present study. Costa et al. (2004) analyzed the growth of the microalgae *Dunaliella viridis* e *T. chuii* in treatments with culture medium Guillard f/2 containing 10, 20, 30 and 40% of seawater. The authors observed higher growth rates of 2.11 divisions day^{-1} with 30% of seawater added for D. viridis, and 2.28 divisions day^{-1} with 40% of seawater added for *T. chuii*. De La Peña and Villegas (2005) evaluated different photoperiod, light intensity and nutrient concentration in cultures of *Tetraselmis tetrathele*, achieving growth rates of 1.4 divisions day^{-1} for the cultures subjected to constant light.

The nitrate reduction in the Guillard f/2 culture medium was statistically faster ($p < 0.05$) than observed in Conway medium, with a mean of 108 ± 12h. Possibly because of this fact, the maximum cell concentration was lower in relation to Conway medium, since the nutrient limitation in this medium was more delayed, thus allowing a greater supply of nutrients to the microalgae, which resulted in a higher productivity ($p < 0.05$), which was 3.38 ± 0.02 g L^{-1} (Figure 2).

For optimal performance of the cultivation, several nutrients are essential for algal development. The so-called macronutrients or essential nutrients are usually found in significant amounts, and the micronutrients are requires in small quantities by the organisms (SIPAÚBA-TAVARES; ROCHA, 2003).

Growth curves and the nitrate concentration curves presented similar behavior, but with some differences. The culture in Conway medium presented a more pronounced exponential phase however entered stationary phase later, after about 120 hours (Figure 2).

Maximum algal biomass (Table 4), both in Guillard f/2 (2.28 ± 0.08 g L^{-1}) and in Conway (3.37 ± 0.03 g L^{-1}), were achieved in early stationary phase and presented significant statistical difference ($p < 0.05$). This occurred as consequence of nitrate uptake by the microalgae, since in this phase of the growth curve were observed significant lower nitrate concentrations ($p < 0.05$), with values of 2.90 ± 0.56 mg L^{-1} in Guillard f/2 medium, and 1.35 ± 0.35 mg L^{-1} in Conway medium.

Morais and Costa (2008) analyzing different bioprocesses for the removal of carbon dioxide and nitrogen oxide by two microalgae in order to use the gases generated during the coal combustion, attained the highest productivities of 1.11 ± 0.14 g L^{-1} for *Spirulina* sp. and 0.98 ± 0.10 g L^{-1} for *Scenedesmus obliquus* in cultivations performed with 6% CO_2 and $NaNO_3$.

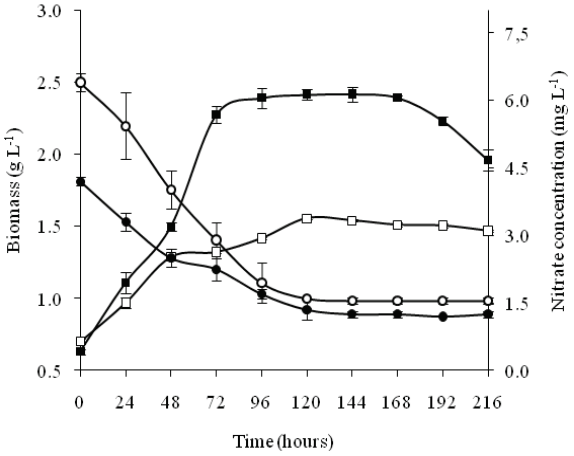

Figure 2. Growth rates (squares) in biomass (g L^{-1}) and depletion curves of nitrate (circles) in the cultivation of the microalgae *T. tetrathele* in the culture media Guillard f/2 (empty symbols) e Conway (filled symbols). Each dot refers to the average of four replicates ± standard deviation.

Parisi et al. (2009) evaluated the production of phenolic compounds by *Spirulina platensis*. The average productivity was 1.49 g L^{-1} in cultivations performed using 2 to 4 g L^{-1} $NaNO_3$ in the culture medium.

According to Lourenço (2006) the nitrate (NO_3^-) is the most stable form of nitrogen present in seawater and possibly the most assimilated by the phytoplankton. After phase exponential, the growth rate tends to stabilize and is always low, when compared to prior phases, with values close to zero, in this way, the duration of this phase depends on the availability of essential nutrients and light.

Table 3. Effect of nitrate depletion on the growth parameters of the microalgae *T. tetrathele* in the two culture media tested.

Culture medium	Culture period (h)	Initial concentration of nitrate (mg L^{-1})	Reduction time of nitrate (hour)	Growth rate (day^{-1})	Algal biomass (g L^{-1})
Guillard f/2	210	6.40 ± 0.20^{a*}	108 ± 12^{a}	0.412 ± 0.06^{a}	2.44 ± 0.07^{a}
Conway	210	5.70 ± 0.10^{a}	132 ± 12^{b}	0.473 ± 0.03^{a}	3.41 ± 0.04^{b}

*Different letters in the columns indicate statistical differences at 5% level.

Table 4. Nitrate concentration in the three different phases of growth curve of the microalgae *Tetraselmis tetrathele* in the two culture media tested.

Growth phase of the culture	Guilard f/2		Conway	
	Nitrate concentration (mg L^{-1})	Algal biomass (g L^{-1})	Nitrate concentration (mg L^{-1})	Algal biomass (g L^{-1})
Early exponential phase	6.40 ± 0.28^{a*}	0.63 ± 0.02^{a}	4.20 ± 0.14^{a}	0.64 ± 0.07^{a}
Early stationary phase	2.90 ± 0.56^{b}	2.28 ± 0.08^{b}	1.35 ± 0.35^{b}	3.37 ± 0.03^{b}
Early senile phase	1.55 ± 0.07^{b}	2.23 ± 0.03^{b}	1.20 ± 0.01^{b}	3.21 ± 0.06^{b}

*Different letters in the columns indicate statistical differences at 5% level.

Conclusion

Cultivation in Conway culture medium presented better results concerning microalgae growth, with a more pronounced exponential phase.

The time for nitrate reduction was shorter for the culture performed in the Guillard medium, but the maximum algal biomass produced was higher in the Conway medium. In both treatments no difference was detected for the growth rate.

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