ISSN 2176-5480

22nd International Congress of Mechanical Engineering (COBEM 2013)
November 3-7, 2013, Ribeirão Preto, SP, Brazil

ALTERNATIVE SOURCES OF NUTRIENS FOR PRODUCTION OF MICROALGAE BIOMASS

Kassiana Ribeiro Dos Santos
André Bellin Mariano
e-mail: kassi.ribeiro@hotmail.com
andrebmariano@gmail.com
Center for Research and Development of Sustainable Energy (NPDEAS), Curitiba, Parana, Brazil.

Abstract. *The high cost of production in the cultivation of microalgae in photobioreactors is a barrier to be overcome, therefore is necessary to search to reduce the cost of cultivation. The objective of this work is to compare the growth of the microalge Scenedesmus sp. being cultivated in CHU medium and in an alternative medium composed mainly of fertilizer. The research approach consisted on growing the microalgae in Erlenmeyer type photobioreactors and producing the growth curves for both media. The comparisons between the two, were made based upon three parameters: dry biomass, absorbance, and total lipid quantity. The experiments were made in triplicates in order to quantify the uncertainty in the measurements. The comparison of the cultivation in both media suggest that the modification of the chemical nutrients by fertilizer did not produce significant changes in the number of cells, dry biomass and lipids. In spite of that, the modification resulted in 20% reduction in the growth medium. In this way, the substitution of conventional nutrients by fertilizers, constitute an interesting alternative for the production of biomass from microalgae leading to a cost reduction.*

Keywords: *microalgae, biofuel, fertilizer, culture medium*

1. INTRODUCTION

Due to human activities, air pollution in urban centers has become one of the major problems of society. Originating mainly from fossil fuels, which are used for power generation, automobiles, and in industries such as the manufacture of cement (Loera-Quezada, 2010) where there is a large generation of emissions of carbon dioxide (CO_2), which is one of the greenhouse gases (GHG). CO_2 emissions have increased by 80% between 1970 and 2004 (Loera -Quezada, 2010).

The replacement of diesel oil for biodiesel allows for cleaner transportation systems, resulting in improved air quality (Sales et al. 2006). Biodiesel can be produced from various raw materials such as vegetable oils, used cooking oils and animal fat, but these materials are not abundant, which limits its production (Prabakaran & Ravidran, 2011). The biodiesel produced by plants require a large planting area, for example, in the United States would require 330 million to 450 million hectares to meet 50% of the energy demands of transportation (Chisti, 2007). Therefore, other sources of raw material such as microalgae are being investigated.

Microalgae are single-celled organisms with a fast growth rate that performing photosynthesis and have a high oil content and rapid biomass production (Minowa et al., 1995). The cultivation of microalgae is relatively simple compared to oilseed crops, they can be grown in smaller spaces and are used as a food supplement for humans and animals (Andrade & Costa, 2008), in cosmetics and pharmaceutical applications.

The Center for Research and Development in Self-sustainable Energy (NPDEAS) in Department of Mechanical Engineering, at Federal University of Paraná, was built in 2008 for the purpose of cultivating microalgae in compact horizontal tubes photobioreactors, with the interest of producing biodiesel with the oil isolated from microalgae. The cultivation of microalgae is a good alternative due to the high biomass production per area of cultivation (Chisti, 2007).

In this work, experiments were performed with the microalgae *Scenedesmus sp,* which is part of the Phylum Chlorophyta. It is one of the most common green algae genres, found mostly in freshwater.

Green algae are responsible for most of the production of molecular oxygen on the planet available from photosynthesis. They accumulate starch within their cells, and contain the pigments chlorophyll a and b, carotenes and xanthophylls, the presence of chlorophyll a end b supports the idea that green algae were the ancestors of plants (Vidotti & Rolemberg, 2004).

The high cost for biomass production from the cultivation of microalgae in photobioreactors are obstacles to overcome. Besides water, the added substrates are critical to the growth of microalgae. Sodium nitrate is the nutrient required in largest quantity and also the more expensive (Morais, 2011).

About two years ago, NPDEAS took a sample of microalgae naturally present in the water supply network. The material was kept in flasks with medium Chu (Chu, 1942), on aeration and constant light. After

ISSN 2176-5480

several cultures it was observed the predominance of Scenedesmus. As it is a microalgae native to the region, it has been used in the experiments NPDEAS.

As already mentioned, in order to make possible technical and economical cultivation of microalgae in photobioreactors for the production of biomass to be used for different purposes, there is a need to reduce costs of nutrients.

Therefore, the aim of this study was to monitor the growth of the microalga *Scenedesmus sp*. in photobioreactors type flask, and the evaluation of the composition of the medium parameters: dry matter, absorbance and total lipids.

Replacing the culture medium of NPDEAS Chu pattern used in all crops, we have developed a culture medium called modified- Chu and alternative with the substitution of urea by sodium nitrate and sodium phosphate (monobasic and dibasic) by superphosphate.

2. MATERIALS AND METHODS

For the preparation of modified -Chu, urea was used as nitrogen source and superphosphate as source of phosphorous (Mello et al. 2010).

Urea is used as a synthetic fertilizer for plants for being 100% organic and containing 46% nitrogen (Urquiaga & Malavolta, 2002). The superphosphate simple has ingredients derived from phosphorus, which can reduce nitrogen losses from the liquid to the gaseous state (Oliveira, Trivelin & Oliveira, 2003).

A mass balance in all replaced reagentes was made, in order to compute the contents of major componentes, nitrogen and phosphorus, which were then added to the modified -Chu.

Changes in the composition of the culture medium were motivated by the lower cost of alternative components to the usual reagents. That these costs are listed in Table 1.

The experiment was conducted in a room heated with temperature variations from 18 to 22 ^{0}C, with lighting photoperiod 12 hours a day and 12 hours per night to match the normal day and night. White fluorescent lamps were used, with an intensity of 2500 lux and aeration provided by a generator that possesses continuous bubbling of 5 L.min^{-1} of air.

The experiments were performed in triplicate, consisting of three conical flasks with medium Chu and three Erlenmeyer flasks with medium changed Chu, each with a final volume of 1.6 L. The Erlenmeyer flasks and all materials used in the preparation of the culture medium (pipettes, deionized water, nozzles, culture media) have previously been autoclaved at 125 ° C for 15 minutes, then cooled to room temperature to prevent possible contamination.

Before preparation of the experiment samples were collected from the inoculum to measure absorbance. This was used to standardize the beginning of the two experiments, which were started with 0.3 absorbance.

Table 1 - Composition of the medium Chu and Modified - Chu

Reagents	Concentration the final medium (gL^{-1})	R$ L^{-1}	concentration the final medium (gL^{-1})	R$ L^{-1}
Urea – $(NH_2)_2CO_4$	—	—	0,087	0,00088
Sodium nitrate - $NaNO_3$	0,25	0,0059	—	—
Calcium chloride dihydrate - $CaCl_2.2H_2O$	0,025	0,00063	0,025	0,00063
Magnesium sulfate heptahydrate - $MgSO_4.7H_2O$	0,075	0,0111	0,075	0,0111
Superphosphate $Ca(H_2PO_4)_2H_2O$	—	—	0,175	0,00012
Dibasic potassium phosphate - K_2HPO_4	0,075	0,00414	—	—
Monobasic potassium phosphate - KH_2PO_4	0,175	0,0079	—	—
Sodium chloride – NaCl	0,025	0,00024	0,025	0,00024

ISSN 2176-5480

EDTA - $C_{10}H_{14}N_2Na_2O_8.2H_2O$	0,05	0,0024	0,05	0,0024
Potassium hydroxide - KOH	0,031	0,0012	0,031	0,031
ferrous sulfate heptahydrate - $FeSO_4.7H_2O$	$4,89\ 10^{-3}$	0,11	$4,89\ 10^{-3}$	0,11
Boric acid - H_3BO_4	$11,42\ 10^{-3}$	0,20	$11,42\ 10^{-3}$	0,20
Zinc sulfate heptahydrate - $ZnSO_4.7H_2O$	$8,82\ 10^{-6}$	0,38	$8,82\ 10^{-6}$	0,38
Manganese chloride tetrahydrate - $MnCl_2.4H_2O$	$1,44\ 10^{-6}$	0,67	$1,44\ 10^{-6}$	0,67
Molybdenum oxide - $Na_2MoO_4.2H_2O$	$1,19\ 10^{-6}$	2,45	$1,19\ 10^{-6}$	2,45
Copper Sulfate pentahydrate -$CuSO_4.5H_2O$	$1,57\ 10^{-6}$	0,04	$1,57\ 10^{-6}$	0,04
Cobalt nitrate hexahydrate -$Co(NO_3)_2.6H_2O$	$0,49\ 10^{-6}$	0,76	$0,49\ 10^{-6}$	0,76
Total		4,64		3,74

* Budget provided by the company DIELAB-Commerce Products Laboratories LTD. In the month of September 2012. Source: The Author

2.1 ANALYSIS

Cells count

The cell count was performed daily by light microscopy at 400x magnification. An aliquot of 1 mL was collected after manual shaking of the Erlenmeyer order to homogenize the cells decanted. The cell count was made using a Neubauer chamber mirror, and a digital counter. Counting was carried out in the quadrant of the middle of the chamber, which contains 25 small squares. This quadrant was chosen for having more divisions, making it easier to count and reducing the risk of counting errors in the presence of high concentrations of cells.

Figure 1 shows the quadrant used and the counting direction. Results are expressed as number of cells mL^{-1}.

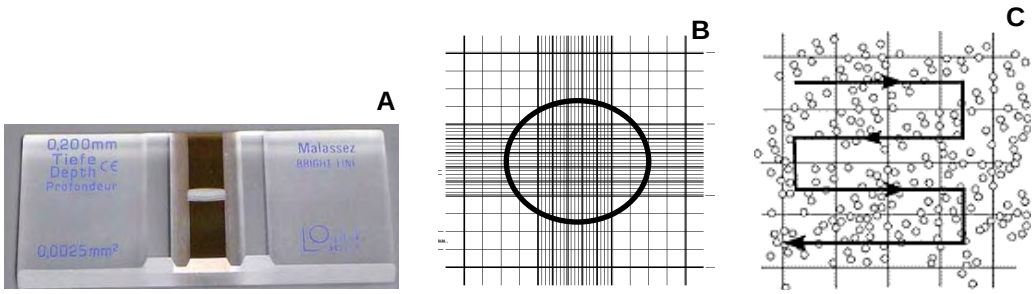

Figure 1 - Schematic of the cell count in a Neubauer chamber. A) Neubauer chamber. B) Representation of a Neubauer chamber viewed under a microscope with 5x magnification. C) Direction of counting in a Neubauer chamber.
Source: A) The Author, B) Villarreal, 2009. C) Villarreal, 2009.

Growth kinetics

For the construction of the growth curve, equation (1) logistics was used, according to Soares (2010). was the profile that best described the experimental growth of the microalgae.

The equation was fitted to the experimental data (cell density) by nonlinear regression, using the program Microsoft Excel 2010, as follows:

ISSN 2176-5480

$$N = \frac{N_{max}}{\left[1+\left(\frac{N_{max}}{N_0}-1\right)^{-\mu t}\right]} \quad (1)$$

wherein:
N = cell density (cells mL^{-1});
N_0 = initial cell density (cells mL^{-1});
N_{max}= maximum cell density (cells mL^{-1});
μ = specific growth rate (cell dia^{-1});
t = cultivation time (days).

Absorbance

The absorbance measurement was performed as an indirect measure of microalgae growth in both culture media. We used the wavelength of 540 nm for the incident radiation, using semi-micro cuvettes with 1.5 ml of polymethylmethacrylate. The blank used was distilled water maintained at room temperature. The measurements were performed on a Lambda 25 spectrophotometer connected to a computer.

The measurement was performed daily from a sample collected from 1.5 ml Erlenmeyer flasks always taking care homogenizing the material.

Dry biomass

The main parameter determined in microalgae cultivation experiments consisted of determining the dry weight through gravimetric method, and the results were represented in gL^{-1}. From this result it was possible to determine the productivity of the culture.

The determination of dry biomass was made every two days by vacuum filtration. Glass fiber filter paper with a pore diameter of 47mm, which was kept in a oven at 60 °C for 24 hours. The papers were kept for 15 minutes in a desiccator with silica under vacuum, to avoid the effect of moisture present in the air, and after weighted using an analytical balance.

Filtration was carried out from 25 mL sample collected from each triplicate studied (standard modified - Chu medium). With the help of a vacuum pump Te-O581, the sample was filtered on pre-weighted paper. The paper with biomass were kept in the same conditions described above and weighted again. The mass of the dried biomass is calculated as the difference between the mass of paper with biomass and paper without biomass.

Determination of lipids

For determination of lipids were collected 500 mL of cultivation and used the method of Bligh and Dyer (1959) adapted for the extraction of lipids from microalgae. This methodology allows us to assess the total amount of neutral lipids present in the sample. This analysis was performed at the end of culture (day 13^o).

The collected sample was flocculated using NaOH. To each 100 mL sample was added 0.5 mL NaOH (Morais, 2011) and stirred for 3 minutes at high speed and 10 minutes at low speeds. It was expected around 20 minutes for the settling of the flocs of microalgae, the supernatant was discarded and the flakes were filtered decanted quantitative role in removing the flocculated part using a vacuum pump. The biomass paper was scraped carefully and then transferred to a petri dish. For total drying in an oven at 60 ° C, dry biomass was manually crushed into a powder microalgae.

A 0.05 g sample of microalgae was weighted and transferred to a polypropylene tube of 12 mL which was called tube 1.

3 mL of chloroform: methanol (2:1, v: v) were added and 10 µL of a solution of butyl hydroxy toluene - BHT (1% in methanol) the tube 1 and the compound subjected to ultrasound in an sonicator for 3 cycles of 15 minutes each. Then the tubes were incubated for 24 hours at 4 °C protected from light with aluminum foil wrapped to avoid photo-oxidation.

Further, the tubes were sonicated again for three cycles every 15 minutes and centrifuged at 5,000 rpm for 5 minutes at 5 °C. The supernatant was recovered with a Pasteur pipette and stored in another tube (tube 2).

1.5 mL of chloroform: methanol (2:1, v: v) were added, in the sedimented biomass tube (1) which was centrifuged again at 5,000 rpm for 5 minutes at 5 ° C. The supernatant was recovered and again transferred to tube 2.

ISSN 2176-5480

2 mL of distilled water were added and 1 ml chloroform to the tube 2. Then, the tube was shaken on a vortex and centrifuged at 5,000 rpm for 10 minutes at 5 °C. The lower phase was recovered with a Pasteur pipette and stored in a glass vial called V1, previously weighed on analytical balance.

To the aqueous phase (second tube) was added 1 mL of chloroform. The tube was then shaken and centrifuged at 5,000 rpm for 10 min. 5 °C, recovering the lower phase and transferring to a glass vial. The vial was left in a laminar flow hood to evaporate the solvent. Then the vial was again quantified its mass.

To determine the total lipid content, we used the gravimetric method. The results were presented as percentage of total lipids per gram of dry biomass.

The fraction of total lipids in % was determined as follows:

$$\text{Lipids} \quad (\%) = \frac{(L_2 - L_1).100}{C} \tag{2}$$

wherein:
L_1 – mass of the empty vial (g);
L_2– mass of the vial with lipids (g);
C – dry biomass (g);

3. RESULTS AND DISCUSSION

Nitrogen has great importance for the development of microalgae to be part of primary metabolism, and phosphorus by making energy exchange and part of the formation of structural molecules (Lourenço, 2006). Was made to exchange sodium nitrate by urea why she has 46% nitrogen (Urquiaga & Malavolta, 2002), and be cheaper.

The microalga *Scenedesmus sp*. was grown for 13 days. During this period were analyzed for dry matter and total lipids, the main analysis of this study in order to define productivity, and secondary analyzes to assess the growth of microalgae, checking the quality of the modified culture medium.

Observed in Figure 2 the result of the monitoring means using the cultivation Chu and modified- Chu by cell counting. The cell density was compared as a function of time, which allows evaluating the speed and the minimum and maximum cell density of the two culture media.

Figure 2 shows the mean result of the two culture media; logistic fit by logistic equation (Eq. (1)) shown in materials and methods section, in growth kinetics that estimates the relative cell growth the time and error bars constituted based ± 2 times the standard deviation of the measurements. Thus the range of values covered by the error bars represent statistically ± 95% of the population (Cumming *et.al*., 2007). It is observed from Figure 3 that the cell density did not change much in the two culture media until the 8 th day. After that day there was a small decline in the cell density of the medium modified- Chu. Thus, it can be said that period was the same for both adjustment means.

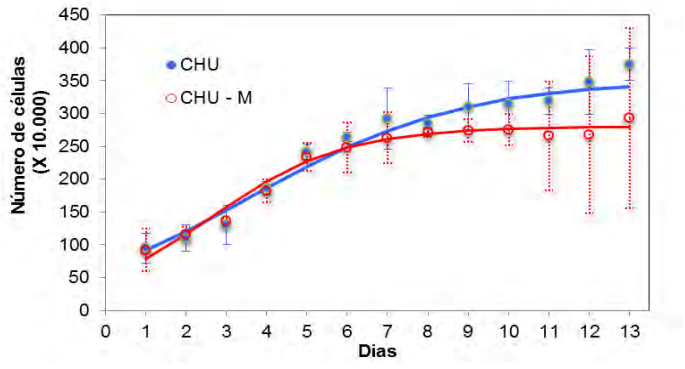

Figure 2 - Growth curve of cell culture media Chu and modified- Chu, Average cell count of Erlenmeyer, adjusting for the logistic and standard deviation.
Source: The Author.

Figure 3 is representing the ratio of the average cell count as measured by absorbance espctrofotometria. This was the optical density incident radiation at a wavelength of 540 nm to show that there is a relationship

ISSN 2176-5480

between absorbance and concentration cell. Can be seen that it is a linear, or can be used to estimate cell number by the value of absorbance. The result of Figure 3 can be used in the laboratory for a rapid estimation of the number of cells in a culture from reading the absorbance and consists of a method widely used in microbiology. Nevertheless, for higher reliability of the results, this estimate was not considered for evaluation of the result as cell density represented in number of cells per mL does not provide information about the amount of biomass produced by the plants. As the focus of NPDEAS consists of bioenergetic efficiency, the quantity of material produced over time provides the information necessary to evaluate the actual productivity of crops. Remember that different microalgae of different sizes can provide similar results in relation to the number of cells in culture, however, different results biomass. Thus, measurements were made of dry biomass crops every two days.

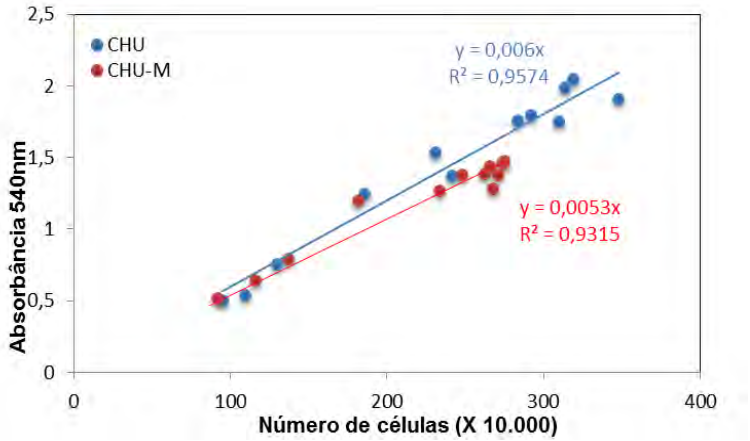

Figure 3 - The relationship between the absorbance at a wavelength of 540 nm and the number of cells in both the culture medium to Chu and modified- Chu.
Source: The Author.

Figure 4 shows the results obtained in the dry biomass gL^{-1} are for the average of three conical flasks of medium modified- Chu. The graph shows the error bars made from the same method as in Figure 2. We can observe that, even after the stabilization of the number of cells from the 7th day of dry biomass crops continues to increase until the 13th day. After 7 days nitrogen levels decrease dramatically (data not shown), and thus the metabolism of amino acids and protein synthesis decreases. All CO_2 fixed by microalgae ends being diverted to the synthesis of carbohydrates and lipids resulting in increased cell size.

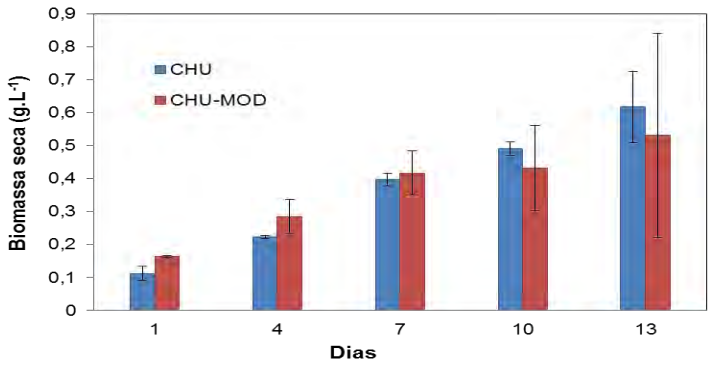

Figure 4 - Increasing dry matter in grams per liter of the medium Chu and modified- Chu
Source: The Author.

When analyzing the total cost to make 1000 L of culture medium, amid Chu would cost R$ 4.64 and modified- Chu R$ 3.74. To determine whether the modified medium is actually more viable than Chu's economic standpoint, an analysis was made of the amount spent per kg medium, which are shown in Table 2.

ISSN 2176-5480

Table 2 - Production cost to Chu and modified- Chu medium biomass concentration in gL^{-1} followed by standard error.

	concentration (g/L)	Price per 1000 L of medium (R$)	Cost of production (R$/kg)
Chu	0,6170 ± 0,108	4,64	7,52
Chu – modified	0,5318 ± 0,249	3,74	7,03

Source: The Author.

Table 2 shows that the cost of the medium modified- Chu is approximately 20% less than the standard Chu means. This result is quite advantageous, especially for biomass production on a large scale, such as in photobioreactors. However, to know if the medium modified- Chu is really productive should compare the two media in terms of lipid fraction according to Eq (2).

Lipid analysis was performed on the last day of culture by the method of Bligh and Dyer, which is based on the use of a monophasic mixture of chloroform, methanol and water (Soares, 2009).

Table 3 shows the results of the amount in percentage of fat that were found by Equation (2). And the value productivity of lipids found in 1000 L of medium means Chu and modified- Chu.

Table 3 - Amount in% of Lipid Biomass in the culture media Chu and modified- Chu.

Means of Cultivation	Amount of Lipids (%)	Lipid Productivity ($mg/L_{\text{de means}}$)
Chu	10,6	65,4
Chu - modified	9,3	49,4

Source: The Author.

The average middle modified- Chu is less. When we make a relationship with the price of the medium Chu and Chu - modified shown in Table 2, we find the following values, the middle Chu 71R$ / $kg_{\text{de lipids}}$ and the modified- Chu 75 71R$ / $kg_{\text{de lipids.}}$

It is important to point that these values are affected by the variability of biomass productivity illustrated in Figure 4 with a standard deviation, for example in the case of using the largest value of productivity, medium Chu cost 60,4 R$/$kg_{\text{de lipids}}$ and medium modified- Chu 51.5 R$/$kg_{\text{de lipids.}}$

4. CONCLUSION

.

The cost found to make the modified culture medium is 20% smaller than the standard culture medium used in NPDEAS. However, due to the large variability observed in lipid productivity, the price of the final product biofuel may vary depending on the range of values covered by the error bars. A future study could be done to reduce variability and optimize growth conditions.

5. ACKNOWLEDGEMENTS

CNPq for financial support. I would like to thank André Mariano Bellin for their help and support during this journey, the team NPDEAS whose support was essential to this research.

6. REFERENCES

Andrade, M. R., & Costa, J. A. (2008).). Cultivo da microalga *Spirulina plantensis* em Fontes alternativas de nutriente. Ciência Agrotec. Pg. 1551-1556.

Chisti, Y. (2007). Biodisel from microalgae. Biotechnology Advances 25. 294 -306.

Chu, S.P (1942) The influence of the mineral composition of the medium on the growth of planktonic algae. J Ecol 30, 284–325.

Cumming, G.; Fidler, F.; Vaux, D. L. (2007). Error bars in experimental biology. The journal of cell biology, Vol. 177, Nº 1, April 9, pág. 7-11. Available in http://jcb.rupress.org/content/177/1/7.full.pdf+html, accessed: September 2012.

Loera-Quezada, M.M. & Olguín, E.J. (2010). Las Microalgas oleaginosas como fuente de

ISSN 2176-5480

biodiesel: retos y oportunidades. Rev. Latinoam Biotecnol Ambiental y Algal, v.1 N_O.1, pág. 91-116.

Mello, T.C.; Gnoatto, V.E.; Morais, K.C.C.; Ribeiro, K.; Miyawaki, B.; Cardoso, A. T. M.; Becker, A.; Pulliam, R.; Vargas, J.V.C. & Mariano, A.B. Efeito econômico da utilização de fertilizantes como substrato no cultivo de microalgas. Available in: http://www.slideshare.net/andrebmariano/artigo-8-efeito-econmico-da-utilizao-de-fertilizantes-como-substrato-no-cultivo-de-microalgas, accessed: june 2011

Morais, K.C.C. (2010) Análise e Desenvolvimento de Aquicultura da Microalga *Phaeodactylum Tricomutum* Em crescimento autotrófico e mixotrófico em fotobiorreatores compactos, Tese de mestrado Universidade Federal do Paraná, Curitiba.

Minowa, T., Yokoyama, S.Y., Kishimoto, M. & Okakurat, T. (1995). Oil production from algal cells of Dunaliella tertiolecta by direct thermochemical liquefaction. Fuel 74, 1735–1738.

Oliveira, P. P. A; Trivelin, P.C.O & Oliveira, W.S. (2003). Eficiência da fertilização nitrogenada com Uréia 15N em *Brachiaria brizantha* cv. Marandu associada ao parcelamento de Superfosfato simples e Cloreto de Potássio. Revista brasileira do solo. vol. 27, núm. 4, 2003, pp. 613-620. Vicosa, Brasil.

Prabakaran, P., & Ravindran, A.D. (2011). A comparative study on effective cell disruption methods for lipid extraction from microalgae. Letters in Applied Microbioloy ISSN 0266-8254.

Sales, J. C., Sombra, A. S. B., Furtado, A. F. G. & Almeida, J. S. (2006). A importância do biodiesel para o meio ambiente, I Congresso de Pesquisa e Inovação da Rede Norte Nordeste de Educação Tecnológica, Natal-RN.

Soares, D., (2010). Avaliação do crescimento celular e da produtividade de lipídeos de microalgas marinhas em diferentes regimes de cultivo. Tese de mestrado Universidade Federal do Paraná, Curitiba.

Vidotti, E. C. & Rollemberg, M. C. (2004). Algas: da economia nos ambientes aquáticos a biorremediação e a química analítica. Química Nova, v.27.

Villarreal, C. M. C. (2009). Operar equipo de labotarorio clinico, camara de neubauer. Available in: http://2l2m-laboratoristaclinico.blogspot.com.br/2009/04/camara-de- http://2l2m-laboratoristaclinico.blogspot.com.br/2009/04/camara-de-neubauer.html accessed: March 2012.

Urquiaga, S. & Malavolta, E. (2002). Uréia: Um adubo orgânico de potencial para agricultura orgânica, cadernos de Ciência & Tecnologia, Brasília, v.19, n.2, p.333-339.

7. RESPONSIBILITY NOTICE

The author(s) is (are) the only responsible for the printed material included in this paper.