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Response of microalga *Haematococcus pluvialis* growing in phototrophic and mixotrophic culture under alternative source of carbon and media

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Abstract

Growth and biochemical composition of microalgae *Haematococcus pluvialis* were evaluated under phototrophic and mixotrophic culture with WC and NPK culture media. Carbon source consisted of sugar cane molasses in mixotrophic cultures. Highest cell density was observed in NPK (4.6 x 10^5 cell mL^{-1}) and WC (3.2 x 10^5 cell mL^{-1}) media under phototrophic culture. Cell density in mixotrophic culture was approximately 22% lower than that in phototrophic culture (p<0.01). High protein (16-54 % dry biomass) and low lipid contents (1.3-10% dry biomass) were obtained in the phototrophic culture of *H. pluvialis*, although amino acid concentrations in mixotrophic culture were high. Results show that phototrophic culture favoured growth of *H. pluvialis* and confirmed that inorganic fertilizer NPK (10:10:10) may replace conventional WC. Mixotrophic culture with sugar cane molasses resulted in high amino acid concentration. The inorganic fertilizer (NPK 10:10:10) and sugarcane molasses may be an alternative nutrient for *H. pluvialis*culture in laboratory conditions.

Keywords: sugar cane molasses, inorganic fertilizers, amino acids.

Introduction

In phototrophic conditions, algal cells absorb energy through luminosity and use CO_2 as a carbon source (Perez-Garcia *et al.,* 2011). Culture in a mixotrophic regime consists of a variation of the heterotrophic one in which CO_2 and organic carbon are simultaneously assimilated while respiration and photosynthesis occur (Lee 2004). The above culture is an important tool to increase the production of several microalgae species (Andruleviciute *et al.,* 2014). Recent research on mixotrophic culture conditions has been underscored due to the fact that organic carbon sources in the absence of or in low luminosity may be associated, with equal or even higher biomass yield than in phototrophic culture conditions (Wang *et al.,* 2013, Matsudo *et al.,* 2015).

High costs in culture media are related to bulk production in microalgae culture in phototrophic and in mixotrophic cultures, normally prepared with solutions featuring analytic grade reagents. Organic sources of carbon may be added in the case of mixotrophic culture, with 50% increase in production costs (Chen *et al.,* 2009). The use of low cost prime matter may be an alternative, with an increase in high nutrition alga biomass production (Dalay *et al.,* 2007). Sugarcane molasses is a prime matter with a high carbon rate and low costs, especially in producing countries such as Brazil. Inorganic NPK fertilizer is also a low cost alternative, with high availability of nitrogen, phosphorus and potassium, relevant for algal development (Sipaúba-Tavares *et al.,*1999).

Haematococcus pluvialis grows well under several culture conditions, such as phototrophic, mixotrophic and heterotrophic regimes (Kobayashi *et al.,* 1992; Hata *et al.,* 2001; Orosa *et al.,* 2005). Several studies have assessed its growth in mixotrophic culture employing carbon sources, especially sodium acetate (Kobayashi *et al.,* 1992; Orosa *et al.,* 2005; González *et al.,* 2009). However, research focusing on optimal levels in the addition of sugarcane molasses, as an alternative source of carbon has not been reported yet.

Current experiment evaluates the response of the *Haematococcus pluvialis* under different culture conditions, with especial reference to its biology, biochemical composition, mainly amino acid profile and effect of the culture water quality on microalgal development.

Materials and methods

Microalgal Strain and Culture Conditions

Haematococcus pluvialis (CMEA 227 C1) was obtained from the culture collection of the Biology Department of the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil. Algae were batch-cultured at 22±1°C and exposed to light at 30 µmol m^{-2} s^{-1}. The two culture media were WC medium (Guillard and Lorenzen 1972) and inorganic fertilizer NPK (10:10:10) (Sipaúba-Tavares *et al.,* 1999). The choice of NPK culture medium at 10:10:10 was based on previous studies on highest cell densities (Scardoeli-Truzzi and Sipaúba-Tavares 2017). Approximately 50g L^{-1} of inorganic fertilizer were added to 1L of distilled water and autoclaved at 1 atm during 30 minutes. Further, 0.75g L^{-1} of sugarcane molasses were added as an alternative source of carbon in the mixotrophic culture of *H. pluvialis* microalgae. The experiment started with 250-mL flasks at a microalgae density of 1 x 10^5 cells.mL^{-1}, cultured in WC media. When cultures reached the late exponential growth phase (14

day), approximately 280 mL of the culture were added in 2-L flasks. The experiment started at density of 0.3 x 10^5 cells.mL^{-1} containing WC medium, and 0.4 x 10^5 cells.mL^{-1}of NPK (10:10:10) medium in phototrophic conditions. In mixotrophic conditions, the experiments started at a density of 0.4 x 10^5 cells.mL^{-1}containing WC medium and with NPK (10:10:10) at density of 0.3 x 10^5 cells.mL^{-1}. Experiments were performed in 2-L volumes with continuous air bubbling. Vitamin B12 complex was added to NPK media at a rate of 0.02 g.L^{-1}, plus biotin (0.01 mg.L^{-1}) (Sipaúba-Tavares *et al.,* 1999). Growth performance and other physiological parameters and analytical methods were reported weekly (1, 7, 14, 21 and 28 days), whereas the composition of amino acid was evaluated at the end of experiment. Only green cells from the exponential growth phase were used as inoculums for the experiment.

Composition and concentration of Sugarcane molasses

Sugarcane molasses, 82.62°BX and pH 5.9, were obtained from Brazilian Molasses Ltda (Brazil). Sugarcane molasses contain 20% water, 8% fructose, 7% glucose and metal ions, such as calcium, potassium, sodium, iron, magnesium, copper, and others. Crude molasses were diluted in distilled water and autoclaved at 1 atm, for 30 minutes. The solution was subsequently used in assays for mixotrophic culture.

Due to growth inhibition of the microalga between 1.5 and 5g. L, six concentrations (0.25, 0.50, 0.75, 1.0, 1.25 and 1.5 g L^{-1}) were selected in current analysis. Microalgae were cultured in 2-L flasks with commercial medium WC under the same conditions described above. Cell growth in all treatments was evaluated in triplicate (n = 3), for 15 days (Figure 1).

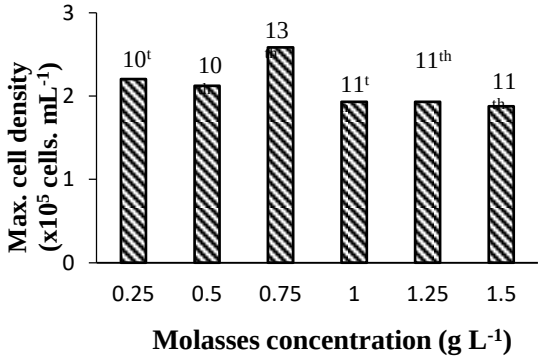

Fig. 1 Maximum cell density of *Haematococcus pluvialis* in relation to sugarcane molasses concentration (10 - 13th day = time in days for maximum cell density).

Growth and Biochemical parameters

Cell growth was monitored during 28 days. Triplicate 1 mL aliquots were removed daily from the microalgae culture and a minimum of 2 x 1 µL^{-1} sub-sample was used for cell quantification, with a Neubauer hemocytometer. Growth rate k (divisions per day) was calculated by the formula: k = (3.322/t2-t1 x log N2/N1) (t = time; N = number of cells. Subscripts denote values at different periods) (Guillard 1973). Doubling time (cell division time or generation time) was calculated from results obtained from growth rate, using the formula: Dt = 1k^{-1}(Guillard 1973). Dry weight was determined weekly, following Vollenweider (1974). Total length (µm), total organic carbon content (TOC) and cell volume were quantified weekly. The total length of 50 specimens was determined with microscope Leica DFC 295 by image analysis system Las Core (LAS V3.8), with a 400X micrometric objective. Cell volume was calculated by mean cell size with the most appropriate geometric form, or rather, the sphere formula (Hillebrand *et al.,* 1999). Total organic carbon was calculated by C = 0.1204.V$^{1.051}$ (C = carbon content in pg.cell^{-1}; V = cell volume), with regression, following Rocha and Duncan (1985).

Total ammonia nitrogen and total phosphorus in the culture media were quantified by spectrophotometer, following Koroleff (1976)and Golterman *et al.,* (1978). Chlorophyll-*a* was extracted with alcohol 90% and quantified at 663 and 750 nm (Nusch, 1980). Biomass was harvested, centrifuged and lyophilized to analyze proteins and lipids (A.O.A.C. 1990).Amino acid (AA) contents were determined by acid hydrolysis with ion-exchange chromatography with high-performance liquid chromatography (HPLC), modified by White *et al.,* (1986). The water of culture media was evaluated weekly; amino acids were assessed at the end of the experiment.

Statistical analysis

One-way ANOVA was applied for simple verification between culture media, with Statistica 10. Tukey's test was appliedwhen differences (p< 0.05) between conditions occurred.

Results

Haematococcus pluvialis in phototrophic culture revealed maximum cell density (3.2 x 10^5 cell mL^{-1}) in commercial medium WC after the 16th day of culture. On the other hand, cell density was higher in NPK medium, with 4.6 x 10^5 cell mL^{-1} on the 20th day. Growth ceased after maximum cell density (p<0.01) was reached in the two media. During exponential growth phase and up to the 12th day, algal biomass was slightly higher in WC culture, whereas alga growth was higher in NPK culture, as from the 13th day. Gradual reduction occurred in mixotrophic culture after maximum cell density. Maximum cell density was reported in WC medium after the 25th day, at 3.6 x 10^5 cel mL^{-1}, and higher in NPK medium (2.4 x 10^5 cell mL^{-1}) on the 19th day. When results for phototrophic culture were compared, cell density of *H. pluvialis* was approximately 22% higher than that in mixotrophic conditions (Fig. 2).

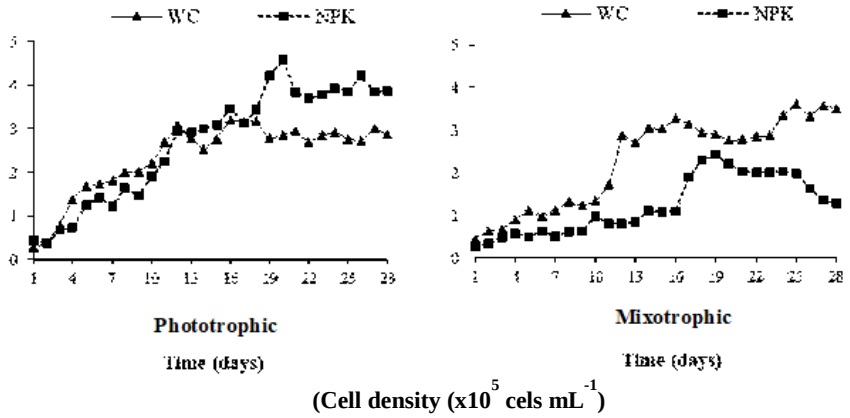

Fig. 2 Growth of *Haematococcus pluvialis* in WC and NPK culture media under phototrophic and mixotrophic culture.

Growth rate and duplication time had better results in phototrophic culture medium, with highest rates in WC medium (k=0.21), affecting a shorter duplication time by 4.83 days. In mixotrophic culture, growth rate and duplication time were slower, whereas variables between phototrophic and mixotrophic cultures were similar in NPK culture. In the case of cell density in WC medium, growth was similar under the two culture conditions. However, it was higher (p<0.05) in NPK medium within phototrophic culture (Table 1). Dry weight of microalgae varied according to culture conditions, with greater rates (p<0.05) in mixotrophic culture, in WC and NPK media. Cell length, chlorophyll-*a* and protein rates were similar (p>0.05) in the two culture media under the two culture conditions (Table 1). Cell volume and total organic carbon rate of *Haematococcus pluvialis* in mixotrophic culture were higher (p<0.05) than in phototrophic culture (Table 1).

Table 1 Parameters of *Haematococcus pluvialis* cultured in WC and NPK media under phototrophic and mixotrophic culture.

Parameters	Phototrophic		Mixotrophic	
	WC	NPK	WC	NPK
Maximum cell density (cell mL^{-1})	3.2 x10^{5b}	4.6 x10^{5a}	3.6x10^{5ab}	2.4 x10^{5c}
Growth rate (k)	0.21	0.18	0.12	0.18
Doubling time (days)	4.83	5.51	8.03	5.63
Dry weight (pg $cell^{-1}$)	1966±1086^c	2798±735^{ab}	2810±822^b	2913±737^a
Total length (µm)	23.7±2^a	21.7±3^a	25.3±1^a	25.7±2^a
Cell volume ($µm^3$)	7707±1976^c	6051±2685^c	9724±1245^b	12044±8314^a
Total Organic Carbon (pg $cell^{-1}$)	1478±400^c	1148±541^c	1892±266^b	2395±1723^a
Chlorophyll-*a* (mg L^{-1})	728±548^a	741±545^a	721±332^a	830±663^a

The values and the mean of three replications and the variation (±) constitute standard deviation. Same letter in the superscript means there were no differences between treatments after ANOVA test (p< 0.05).

Total lipid contents of *H. pluvialis* ranged between 1% and 9.6% of dry biomass in the two cultures. Maximum rate (9.6%) occurred in phototrophic culture in WC medium, whilst minimum content (1%) was

registered in mixotrophic culture in the two culture media. Low lipid rates in alga biomass were affected by high nitrogen concentrations in the media. In fact, WC and NPK media, especially ammonium nitrogen, are rich in nitrogen. Lipid content was lower (below 3% dry biomass) in mixotrophic culture when compared to that in phototrophic culture, averaging 6% dry biomass (p<0.05), due to the addition of molasses to the culture medium (Fig. 3).

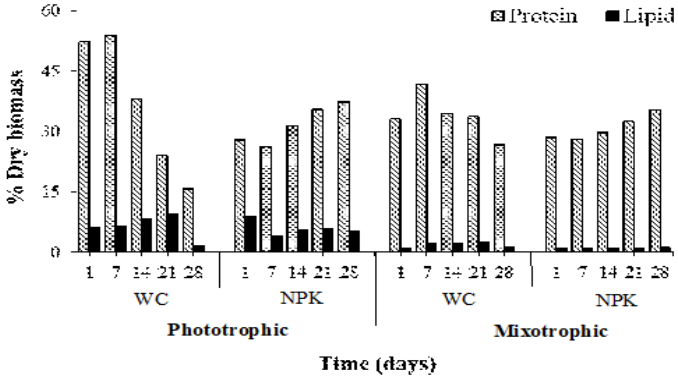

Fig. 3 Weekly variations of proteins and lipids (% dry biomass) contents in WC and NPK culture media under phototrophic and mixotrophic culture.

Haematococcus pluvialis contains high levels of essential (EAA) and non-essential (NEAA) amino acids (Fig. 4). Amino acid contents varied among culture conditions, with the greatest production of total amino acids in mixotrophic culture within NPK medium, with approximately 37 g. 100g dry biomass (p<0.05). Although production of total amino acids was low in WC culture medium, with approximately 15 g. 100g dry biomass in phototrophic culture (p<0.05), the addition of molasses in mixotrophic culture increased the production of total amino acids, almost doubling the contents (28 g. 100g dry biomass) (Fig. 4).

Leucine in EAA was predominant in the two cultures (p<0.05), whilst cysteine occurred in lower concentrations and did not go beyond 1% of total amino acid production (p<0.05). Glutamic acid and alanine in NEAA occurred in greater concentrations for both culture conditions, averaging 20% of total AA production (p<0.05). On the other hand, hydroxyproline had the lowest concentrations (below 3% of total AA production) for dry biomass under culture conditions employed (Fig. 4).

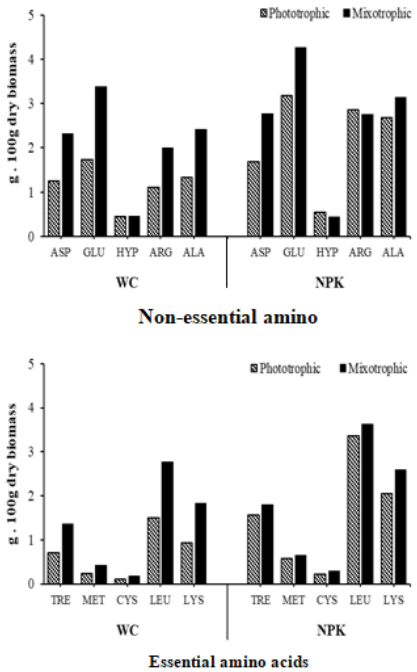

Fig. 4The amino acid profile of*Haematococcus pluvialis* (g. 100g dry biomass) in WC and NPK culture media under phototrophic and mixotrophic culture, where: ASP = Aspartic acid; GLU = Glutamic acid; HYP = Hydroxyproline; ARG = Arginine; ALA = Alanine; TRE = Threonine; MET = Methionine; CYS = Cysteine; LEU = Leucine and LYS = Lysine.

The addition of molasses increased TAN levels in phototrophic culture, varying between 675.5 and 1237.1 mg L^{-1}; and in mixotrophic culture varying between 1316.9 and 3241.6 mg L^{-1} respectively for WC and NPK culture (p<0.05). The uptake of TAN in phototrophic and mixotrophic culture showed higher TAN consumption in WC medium. TAN uptake in NPK medium was low in phototrophic culture and higher in mixotrophic culture, due to increase in nitrogen levels caused by the addition of molasses (Fig. 5).

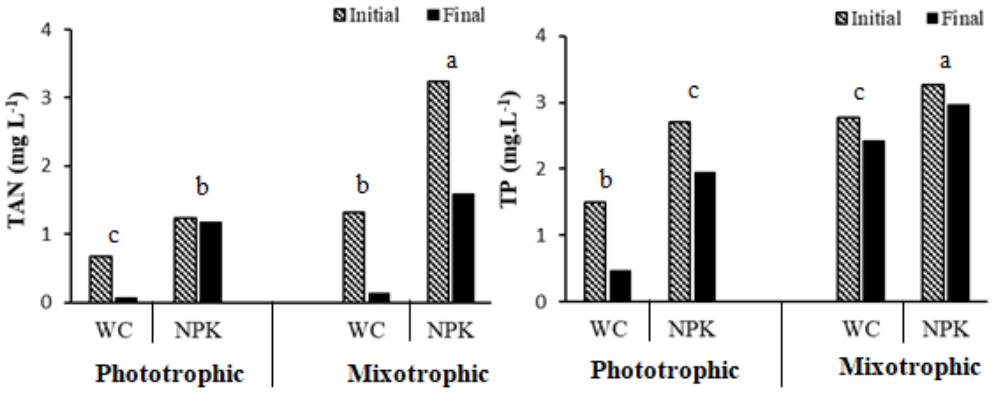

Fig. 5 Total concentration of ammonia nitrogen (TAN) and total phosphorus (TP) in different culture media, under phototrophic and mixotrophic culture. Different letters at the top of each column indicate significant differences (p <0.01).

Initial TP levels were high in phototrophic culture. Highest concentrations occurred in NPK medium (2.7 mg L^{-1}) when compared to that of WC (1.5 mg L^{-1}). The addition of molasses in mixotrophic culture raised TP concentrations for WC and NPK media, 2.7 and 3.2 mg L^{-1} (p<0.01), respectively. The highest TP consumption rate was registered in phototrophic culture for both culture media. Results show that culture conditions and culture media affected nutrient uptake (Fig. 5).

Discussion

The growth of *Haematococcus pluvialis* in WC commercial medium was low when compared to that in NPK 10:10:10 medium in phototrophic culture, albeit higher than that in mixotrophic culture. Comparative studies on phototrophic and mixotrophic cultures of *H. pluvialis* reported better production in the latter (Kobayashi *et al.,* 1992; Orosa *et al.,* 2005; González *et al.,* 2009). High cell densities of microalgae occurred when cultivated in mixotrophic culture due to supplementation of carbon organic sources (Liang *et al.,* 2009; Lee 2001). Andruleviciute *et al.,* (2014) reported better results for *H. pluvialis* in mixotrophic culture when 2 g L^{-1} glycerol were employed and when compared to phototrophic culture in BG-11 medium, with highest productivity rates 2.33 and 1.48 g L^{-1}, respectively. However, in their study with chlorophyceae, Wan *et al.,* (2011) reported reduced cell density under mixotrophic conditions of culture, similar to that in current study in inorganic fertilizer NPK-based (10:10:10) medium.

Variations occurred in growth rate and dry weight during the experimental period when phototrophic and mixotrophic culture were compared. González *et al.,* (2009) attributed variations to algal characteristics, such as microalgae strain and concentration of organic substrate in mixotrophic culture, due to increase and decrease of parameters. Differences in cell density and growth rate in mixotrophic culture may be attributed to organic substrate´s impurities, inhibition or availability (Liang *et al.,* 2010). Sterilized *in natura* molasses were employed in current analysis without the removal of impurities or any other treatment. Methods that favor substrate´s availability, such as ion exchange resins, increase biomass production (Liu *et al.,* 2013).

A longer adapting time was required when algal cells were cultivated in new substrates so that their own assimilation transport could be developed (Perez-Garcia *et al.,* 2011). Low assimilation bysubstrate may be related to preference for absorption of another substrate (Narang and Pilyugin 2005). In current analysis, low cell density under mixotrophic culture in NPK medium was due to the preference of the microalgae*Haematococcus pluvialis* for a substrate with more absorption capacity, making difficult the uptake of the alternative one.

Culture conditions and nitrogen sources affect the growth of microalgae. Nitrogen and total phosphorus requirements are different for phototrophic and mixotrophic cultures (Alkhamis and Qin 2015). Nitrogen and total phosphorus uptake is greater in phototrophic culture than in the mixotrophic one (Kim *et al.,* 2013). In current study, N and P consumption by *H. pluvialis* featured similar characteristics, or rather, consumption was greater in WC medium under phototrophic culture.

Nutrient concentrations in culture media affect algae growth and their biochemical composition. Although a greater protein synthesis rate has been reported when microalgae are cultivated in nitrogen-rich media, nitrogen restriction decreases lipid accumulation (Martínez *et al.,* 2000; Adesanya *et al.,* 2014). Current analysis showed that phototrophic culture featured high protein synthesis and low lipid accumulation. However, the addition of molasses as substrate in mixotrophic culture decreased protein and lipid contents of *H. pluvialis*. The uptake of organic substrates decreases protein and lipid accumulation and all enzymatic activities by at least 20% (Reed *et al.,* 2010).

Studies on amino acid evaluation, especially for *H. pluvialis*, are scarce. In fact, the species has high essential and non-essential amino acid production, such as leucine and glutamic acid and low production of methionine, histidine and cysteine. In most microalgae species, leucine and glutamic acid make up most of total amino acids, whereas methionine, histidine and cysteine feature low rates (Brown 1991). Lorenz (1999)analyzed the microalgae species and obtained similar patterns of amino acid synthesis, with great production of leucine (1.84 g 100g) between EAA and glutamic acid (2.39 g 100g), and alanine (1.92 g 100g) between NEAA. However, concentrations were lower than those obtained in current study with mixotrophic and phototrophic cultures, with the exception of WC medium in phototrophic culture.

The production of total amino acids in *Haematococcus pluvialis* may be stimulated by adding organic carbon sources, such as sugarcane molasses. In NPK medium (10:10:10), the production of total amino acids in mixotrophic culture increased dry biomassfrom 31.28 g to 36.56 g. 100g, whereas increase rate was higher in WC medium, or rather, from 15.14 g to 27.90 g. 100g dry biomass. Ji *et al.* (2014) also underscored that the addition of an organic carbon source increased the production of total amino acids in Chlorophyceae microalgae. The addition of monosodium glutamate provided a maximum production of amino acids of 50.39 g 100g in mixotrophic conditions, whereas production reached 37.12 g 100g in phototrophic conditions.

Sugarcane molasses as an alternative source for organic carbon in *H. pluvialis*culture may be recommended to increase the production of total amino acids without high productioncosts. This is due to its great availability in several countries, including Brazil. Sugarcane molasses is an economically viable alternative to replace high cost prime matters, such as sodium acetate and glucose, and others usually used in mixotrophic cultures. Its employment should be assessed since increase in biomass production is also affected by the culture medium.

Conclusion

MicroalgaeHaematococcus *pluvialis* featured greater cell density in phototrophic medium. NPK medium 10:10:10 has been recommended due to greater cell density in *H. pluvialis* phototrophic culture. Culture medium in mixotrophic culture directly affected cell density, with production decrease (NPK) or increase, as has been reported in WC medium. Although the addition of molasses failed to affect physical and chemical variables of the culture medium, it increased the production of amino acids. High cost compounds, such as protein and lipids, are greater in phototrophic culture. In the production of the microalgae, culture condition should be taken into account according to aims proposed. Inorganic fertilizer (NPK 10:10:10) and sugarcane molasses would represent an alternative for the culture of *H. pluvialis* in laboratory conditions.

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