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Original Article

Effect of untreated and pretreated sugarcane molasses on growth performance of *Haematococcus pluvialis* microalgae in inorganic fertilizer and macrophyte extract culture media

Efeito do melaço da cana-de-açúcar pré-tratado e não-tratado no crescimento da microalga *Haematococcus pluvialis* em meio de cultura com fertilizante inorgânico e extrato de macrófita

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Abstract

The growth of *Haematococcus pluvialis* in two alternative culture media NPK (10:10:10) and ME (macrophyte extract), under mixotrophic conditions using sugarcane molasses as a carbon source were evaluated for 28 days. The molasses was used in two different ways, in a native form (untreated) and a hydrolyzed (pretreated). Cell density of *Haematococcus pluvialis* in mixotrophic cultivation was higher in pretreated molasses. Growth rate was higher when pretreated molasses were employed in mixotrophic cultivation with NPK culture medium (k=0.5 7th growth day). Biomass, chlorophyll-*a*, conductivity and total inorganic nitrogen were not significantly different (p>0.05) during the experimental period for two mixotrophic cultivation and culture media. Protein contents of *H. pluvialis* biomass were higher in NPK culture medium with pretreated molasses (50% dry biomass). Annual biomass production was 520 kg^{-1} dry biomass with untreated molasses for two culture media, and 650 and 520 kg^{-1} dry biomass with pretreated molasses for NPK and ME culture media, respectively. The use of NPK and ME culture media in mixotrophic cultivation may be a new protocol for *H. pluvialis* cultivation due to the low cost and similar annual production.

Keywords: *Eichhornia crassipes*, NPK, mixotrophic condition, biological data.

Resumo

O crescimento de *Haematococcus pluvialis* foi avaliado em dois meios de cultura alternativos NPK (10:10:10) e ME (extrato de macrófita), em condições mixotróficas utilizando melaço de cana-de-açúcar como fonte de carbono durante 28 dias. O melaço foi utilizado de duas maneiras diferentes, bruto (não tratado) e hidrolisado (pré-tratado). A densidade celular de *Haematococcus pluvialis* em cultivo mixotrófico foi maior em melaço pré-tratado. A taxa de crescimento foi maior no cultivo mixotrófico pré-tratado em meio de cultura NPK com k=0,5 (7º dia). Biomassa, clorofila-*a*, condutividade e nitrogênio inorgânico total não foram significativamente diferentes (p>0,05) durante o período experimental para as duas condições mixotróficas e meios de cultura. Os teores de proteína de *H. pluvialis* foram maiores no meio de cultura NPK em melaço pré-tratado, acima de 50% da biomassa seca. A produção anual de biomassa foi de 520 kg^{-1} de biomassa seca em melaço não tratado para os dois meios de cultura e de 650 e 520 kg^{-1} de biomassa seca em melaço pré-tratado para meios de cultura NPK e ME, respectivamente. O uso de meios de cultura NPK e ME em cultivo mixotrófico pode se tornar um novo protocolo adotado para o cultivo de *H. pluvialis* devido ao baixo custo e similar produção anual de biomassa.

Palavras-chave: *Eichhornia crassipes*, NPK, condição mixotrófica, dados biológicos.

1. Introduction

Microalgae cultivation represents a valuable source of many natural products with diverse application in the industry and depends on many elements such as vitamins and nutrients (potassium, nitrogen and phosphorous). Thus, the culture medium is the main factor that increases the microalgae cultivation. The use of compounds that reduce the costs for maintaining high algal biomass production with high nutritional value, is an alternative that have been investigated carefully for microalga cultivation. Among alternatives products for microalgae cultivation may be mentioned the inorganic fertilizer (NPK) and extract from the macrophyte *Eichhornia crassipes* (ME)

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(Sipaúba-Tavares et al., 2009, 2015, 2020). Nitrogen and phosphorous from agriculture fertilizer are important nutrients for biomass production and promote great influence on microalgal growth through direct impact on the photosynthetic apparatus (Li et al., 2019). Macrophytes are rich in nutrients, fiber, amino acids, and absorb high amounts of nutrients from environment (mainly nitrogen and phosphorous) during the growing season or for achieving luxury uptake (Rather and Nazir, 2015).

The cultivation condition is another factor that influences the microalgae growth. Some species can adapt their growth from photoautotrophic to mixotrophic cultivation (combination of organic nutrition and light) which promote high growth rate and biomass production (Kadkhodaei et al., 2015). In general, microalgae cultivation has been carried out mainly under photoautotrophic condition using light as energy and CO_2 as carbon source. Mixotrophic cultivation is a suitable method for microalgae species that are not able to grow in complete darkness and most microalgae species produce higher biomass and other biochemical products when many nitrogen sources are available (Lage et al., 2019). In this condition microalgae assimilate CO_2 and organic carbon simultaneously, however light is also required (Wen et al., 2019).

The growth of some microalgae species becomes limited and mixotrophic cultivation has been suggested for using light and chemical energy to increase the biomass concentration. When the autotrophic and heterotrophic metabolism are integrated the reduced carbon source available in the culture medium is oxidized. The main substrates used for mixotrophic growth of microalgal species are glucose, ethanol, acetate or glycerol (Cecchin et al., 2018). Glycerol and glucose are commonly added as organic carbon sources and, are highly effective in increasing the microalgae production under mixotrophic conditions. However, they have a high cost representing about 80% of the culture medium total cost (Yee, 2015). Alternatively, sugarcane molasses has been identified as a promising carbon source for microalgae cultivation because its production is well adapted to tropical climates and contains a high level of sugar (Liu et al., 2013).

Abundant nutrients, total sugar (mainly sucrose, glucose and fructose), water, crude protein, fat, heavy metals and vitamin are present in molasses, which can be used after pretreatment or even in its native form (untreated) (Dong et al., 2020). The use of native molasses (untreated) as carbon source reduces the cost in the microalgae cultivation under mixotrophic conditions. It promotes the increase in the reduced sugars concentration, converting sucrose into glucose and fructose (Mondal et al., 2017). Besides this, pretreatment such as hydrolysis, is usually necessary for the raw substrate to be used in mixotrophic culture, due to algal cells being usually unable to directly utilize the components (Wang et al., 2018).

Haematococcus pluvialis is an unicellular green microalga characterized a good resource of protein and is currently the major producer of a natural pigmentation (astaxanthin), and is able to fix exogenous carbon source (Sipaúba-Tavares et al., 2015; Shao et al., 2019). The current study investigated whether untreated and pretreated molasses are suitable for *H. pluvialis* growth and the influence of alternatives culture media such as inorganic fertilizer and macrophyte extract. Analyses evaluates whether: (1)- sugarcane molasses may be employed as an external source of carbon when *H. pluvialis* is cultured in NPK and ME culture media to enhance biomass production; (2)- there are differences in the use of sugarcane molasses in its native form (untreated) and hydrolyzed (pretreated) in the growth performance of *H. pluvialis*; (3)- NPK and ME culture media may present profitable economic indicators for *H. pluvialis* production in mixotrophic conditions.

2. Materials and Methods

2.1. Microalgal strain and culture conditions

The green microalga *Haematococcus pluvialis* (CMEA 227 C1) was cultured in phototrophic condition in batch-culture on WC medium at 22 ± 2°C and exposed to light intensity of 30 µmol photons m^{-2} s^{-1} with 24 hours light cycle and bubbled with constant aeration to maintain dissolved oxygen concentration at 7.2 ± 0.1 mg L^{-1}. The experiment starts with 10 mL culture volume with a density of 0.3 x 10^5 cells mL^{-1} in WC culture medium, which was transferred to 250 mL (Sipaúba-Tavares et al., 2015). At the end of the exponential growth phase (7^{th} day) the culture was transferred to 2 L flasks with NPK culture medium at a density of 1 x 10^5 cells mL^{-1}. After the 7^{th} growth day, the culture medium at a density of 0.7 x 10^5 cells mL^{-1} was transferred to 2 L experimental recipients containing the culture media NPK (10:10:10) and ME (macrophyte extract, *Eichhornia crassipes*) under two mixotrophic cultivation conditions (untreated and pretreated sugarcane molasses). Cell growth was evaluated in triplicate (n=3) for 28 days, in all treatments. Only green cells were used as inoculums for the experiment (Figure 1).

2.2. Experimental conditions

On of the alternatives culture media used was composed by an inorganic fertilizer (NPK 10:10:10) (Scardoeli-Truzzi and Sipaúba-Tavares, 2017) and the other by macrophyte extract (ME - *Eichhornia crassipes*) (Sipaúba-Tavares et al., 2009) using sugarcane molasses in its native form (untreated) and hydrolyzed (pretreated). The NPK culture medium was composed of approximately 50 g L^{-1} of NPK (10:10:10) dissolved in a 1 L of distilled water, and each flask used 40 mL of the medium. ME was obtained using the aquatic plant *Eichhornia crassipes*. Approximately 5 kg (wet weight) of plants were washed gently in tap water to remove detritus and epiphytes. The plant material was initially dried in the sun and subsequently in an oven at 60°C for 24 hours. The dried plant material was homogenized in a grinder and boiled in distilled water for one hour. The hot macrophyte extract was filtered and autoclaved at 120°C for 20 minutes. A 70 mL sample was collected and cooled. It was diluted with stilled water up to 1.4 L, when 2.5 mL NPK were added. In both culture media (NPK and ME) vitamin B_{12} complex was added, and the ingredients and composition of nutrients are shown in Table 1.

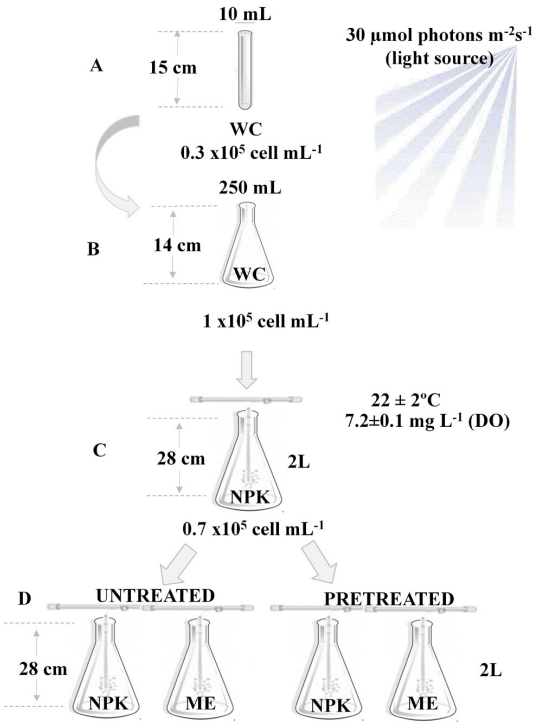

Figure 1. Diagram of *Haematococcus pluvialis*, where: (A) maintenance of strain in 10 mL with WC culture medium; (B) initial culture in 250 mL with WC culture medium; (C) culture in 2 L with NPK culture medium; (D) experiment of mixotrophic cultivation with untreated and pretreated sugarcane molasses with two different culture media, NPK and ME (macrophyte extract).

Table 1. Nutrient concentrations and chemical composition of NPK (10:10:10) and ME (macrophyte extract – *Eichhornia crassipes*) culture media.

Chemical	Culture Media	
Composition	**NPK**	**ME**
Nitrogen (mg L^{-1})	105	95
Phosphorus (mg L^{-1})	107	91
Potassium (mg L^{-1})	107	56
Magnesium (µg L^{-1})	0.008	3
Iron (µg L^{-1})	0.0006	2,130
Calcium (µg L^{-1})	0.135	1
Copper (µg L^{-1})	0.0001	0.05
Vit B_1 (mg L^{-1})	7	7
Vit B_2 (mg L^{-1})	7	7
Vit B_6 (mg L^{-1})	5	5
Vit B_{12} (µg L^{-1})	33	33
Vit H (mg L^{-1})	10	10

2.3. Sugarcane native (untreated) and hydrolyzed (pretreated) molasses

Sugarcane molasses, 82.62°BX and pH 5.9, was obtained from Brazilian Molasses Ltda (Brazil). Sugarcane molasses contain 20% water, 8% fructose, 7% glucose and mineral ions, such as calcium, potassium, sodium, iron, magnesium, copper, and others. The native (untreated) molasses was diluted in distilled water. For the experiments using hydrolyzed (pretreated) molasses, 0.4% (w/w) of acid solution (3M HCl) was added to the native molasses, incubated for 20 minutes at 80°C so that the sucrose could be completely hydrolyzed to glucose and fructose. Subsequently, the hydrolyzed molasses was filtered in Whatman filter paper (diam. 47mm and 0.5 mm pore) using a vacuum pump, for retention of possible impurities. All molasses were autoclaved at 1 atm, for 30 minutes and the solutions were subsequently used in assays for mixotrophic cultivation. About 0.75 g L^{-1} of molasses was added to each culture flask (Scardoeli-Truzzi and Sipaúba-Tavares, 2017).

2.4. Cell growth, biochemical of microalgae and parameters of culture media

The cell density was monitored for 28 days, 1 mL aliquot was removed daily from the microalgae culture media in triplicate and a minimum of 2 x 1 µL^{-1} sub-sample was used for cell quantification, with a Neubauer hemocytometer. Growth rate (k) was calculated by $k = (3.322/ t_2\text{-}t_1 \times \log N_2/N_1)$, where: t = time, N = number of cells; subscripts denote rates at different times (Guillard, 1973). Doubling time (cell division time or generation time) was calculated from results obtained from growth rate, by $DT = 1k^{-1}$ where: DT = doubling time and $1k^{-1}$ = division per days (Guillard, 1973). Dry biomass was measured weekly by filtration of the sample using 0.45 µm glass fiber filters, following Vollenweider (1974). Chlorophyll-*a* concentration was collected weekly and determined by extracting pigments with alcohol 90% and spectrophotometer readings (663 and 750 nm) and was processed according to the methodology presented by Nusch (1980). Dissolved oxygen, pH and conductivity were measured weekly with a multiparametric probe YSI 556 MPS. Total phosphorous (TP) and total inorganic nitrogen (TIN) in the samples were quantified by spectrophotometry following Golterman et al. (1978) and Koroleff (1976). Lipid, protein, nitrogen and carbon contents of microalgae biomass was harvested, centrifuged and lyophilized. Lipid contents were extracted with a petroleum ether and were quantified gravimetrically (AOAC, 2012). The protein, nitrogen and carbon levels of the microalgae were measured by Dumas's combustion method, supplied by Leco (CN628). Biochemical composition of microalgae was expressed in percentage of dry biomass (%)

2.5. Economical aspects

Economic evaluation was performed considering a productive unit with 100,000 L^{-1} capacity, with 28 days cultivation period. The culture media components cost includes the prices of sugarcane molasses (untreated and pretreated), NPK (10:10:10), *Eichhornia crassipes* (ME) and B vitamins. The cost for macrophyte acquisition were considered null, due to the high availability of *Eichhornia crassipes* in this region. Thus, the cultivation in ME culture medium considered only costs for B complex vitamins and inorganic fertilizer (NPK) and sugar cane molasses additions.

2.6. Statistical analysis

The microalgae biochemical composition was evaluated at the beginning and end of the experiment, and it was possible to verify in terms of percentage the increments of protein, lipid, nitrogen and carbon. The average values and standard deviations (SD) were calculated for microalgae variables and culture media, which results were shown in mean ± SD. Significant differences were determined using the method of analysis of variance (One-way ANOVA) with Statistica 10.

3. Results

Potassium of NPK culture medium was almost twice as higher than ME, and N and P were also higher. However, in ME culture medium, Mg, Fe, Ca and Cu presented values from two to three times higher. The highest difference was observed in Fe with 2,130 µg L^{-1} in ME and 0.0006 µg L^{-1} in NPK culture medium (Table 1).

Cell density of *Haematococcus pluvialis* in mixotrophic cultivation was higher in pretreated molasses than untreated molasses. In ME culture medium the cells concentration was below 2.4 x 10^5 cell mL^{-1} (19th day) and in NPK culture medium was below 4 x 10^5 cell mL^{-1} (21st day) both in pretreated molasses cultivation. Under untreated molasses condition, the density of *H. pluvialis* in NPK culture medium was only higher after 17th growth day with cell density 2.5 x 10^5 cell mL^{-1} than ME culture medium with 1.4 x 10^5 cell mL^{-1} (9th day). However, in ME culture medium from the 17th growth day, cell density decreased and remained below 1.1 x 10^5 cell mL^{-1} (21st day). Growth rate was higher in pretreated mixotrophic cultivation in NPK culture medium with k=0.5 (7th day), although in untreated sugarcane molasses, the highest k was observed in ME culture medium (k=0.4) (Figure 2).

Biomass, chlorophyll-*a*, conductivity and TIN were not significantly different ($p>0.05$) during the experimental period for two mixotrophic cultivation and culture media. However, doubling time, pH and TP were higher in NPK culture media. Doubling time was above 5.6 ± 0.5 days (NPK – untreated) and biomass dry weight of *H. pluvialis* was around 0.4 to 0.6 g L^{-1}. Mean cell density was higher in the pretreated molasses with 4 x10^5 cells mL^{-1} in NPK and 2.4 x 10^5 cell mL^{-1} in ME culture medium. Chlorophyll-*a* contents were higher in NPK culture medium ranging between 0.9 ± 0.6 mg L^{-1} to 1.8 ± 0.7 mg L^{-1} to NPK and for ME was below 0.6 ± 0.1 mg L^{-1} in the two molasses treatments (Table 2).

The pH was alkaline in culture media and conductivity was above 717 ± 34 µS cm^{-1} (ME – pretreated). For TIN and TP were high in NPK culture medium, where TIN was above 1.9 ± 0.9 mg L^{-1} (pretreated) and TP above 3.2 ± 0.1 mg L^{-1} (untreated). TIN and TP in ME culture medium were below 0.5 ± 0.4 mg L^{-1} (pretreated) and 0.8 ± 0.2 mg L^{-1} (untreated), respectively (Table 2).

Protein, lipid and nitrogen contents of *H. pluvialis* biomass were higher in NPK culture medium in pretreated molasses, with protein higher than 50%, lipid 5.6% and nitrogen 7.7% of biomass dry weight. The highest carbon contents of *H. pluvialis* biomass was reported in ME culture medium in untreated molasses with 56% biomass dry weight, in general carbon contents were above 45% biomass dry weight in NPK culture medium in untreated molasses (Figure 3).

Total annual cost for culture media composition was higher in NPK culture medium with US$ 12,350 for untreated molasses and US$ 13,325 for pretreated molasses. However, ME culture medium presented lower cost with US$ 5,980 in untreated molasses and US$ 6,955 in pretreated molasses. The annual biomass production was similar for both culture media and mixotrophic condition,

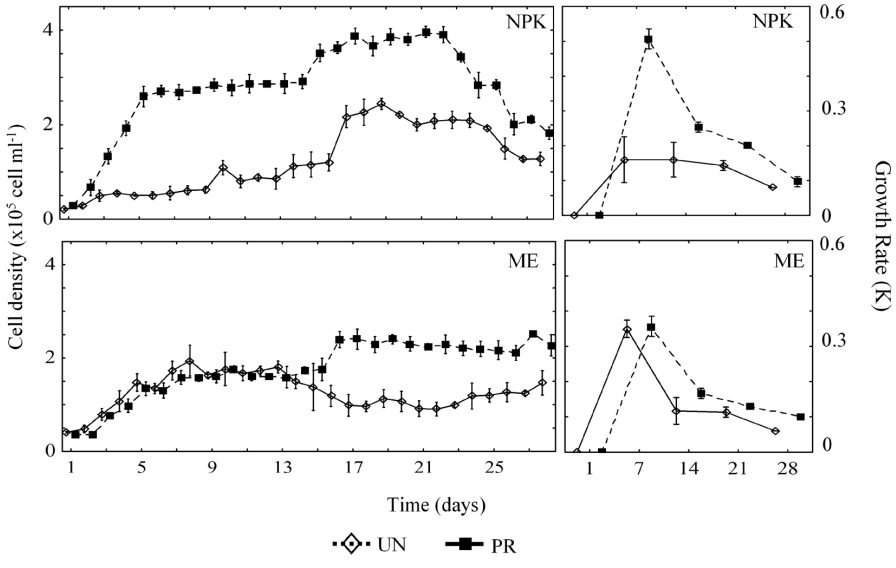

Figure 2. Cell density and growth rate of *Haematococcus pluvialis* in two different culture media NPK and ME, in mixotrophic cultivation untreated (UN) and pretreated (PR) sugarcane molasses. Error bars express standard mean deviations.

Table 2. Mean and standard deviation of *Haematococcus pluvialis* microalgae and culture media (NPK and ME) parameters measured in mixotrophic cultivation (untreated and pretreated sugarcane molasses). ANOVA (test) between untreated and pretreated sugarcane molasses cultivation.

Parameters	Culture media					
	NPK			ME		
	Untreated	Pretreated	Anova	Untreated	Pretreated	Anova
Microalgae						
MCD	2.4 x 10^5	4.0 x 10^5	-	1.9 x 10^5	2.4 x 10^5	-
DT	5.6 ± 0.5	5.9 ± 0.5	p<0.05	5.7 ± 0.1	6.0 ± 0.2	ns
BDW	0.4 ± 0.02	0.5 ± 0.01	ns	0.4 ± 0.06	0.4 ± 0.04	ns
Chlo-*a*	0.9 ± 0.6	1.8 ± 0.7	ns	0.4 ± 0.2	0.6 ± 0.1	ns
Culture Media						
pH	7.4 ± 0.2	7.7 ± 0.2	p<0.05	8.5 ± 0.1	8.5 ± 0.1	ns
Cond	844 ± 90	812 ± 109	ns	762 ± 32	717 ± 34	ns
TIN	3.0 ± 0.8	1.9 ± 0.9	ns	0.4 ± 0.4	0.5 ± 0.4	ns
TP (mg L^{-1})	3.2 ± 0.1	3.5 ± 0.3	p<0.05	0.8 ± 0.2	0.7 ± 0.05	ns

MDC = mean cell density (cell mL^{-1}); DT = doubling time (days); BDW = biomass dry weight (g L^{-1}); Chlo-*a* = chlorophyll-*a* (mg L^{-1}); Cond = conductivity (µS cm^{-1}); TIN = total inorganic nitrogen (mg L^{-1}); TP = total phosphorous (mg L^{-1}); - = not applicable; ns = not significant difference.

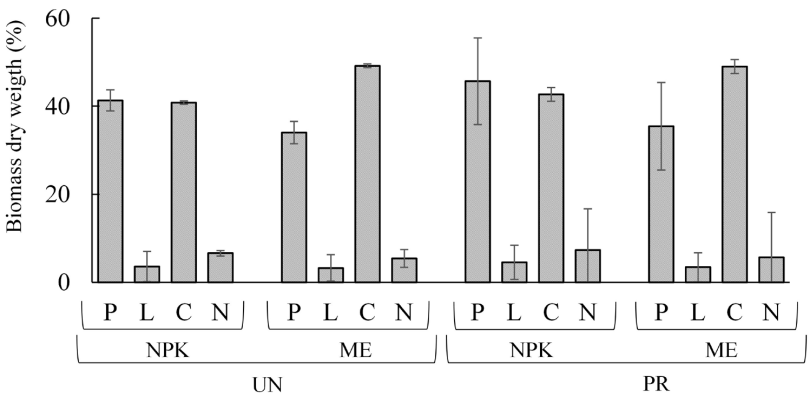

Figure 3. Protein (P), lipids (L), carbon (C) and nitrogen (N) (% biomass dry weight) of *Haematococcus pluvialis* growth in two different culture media (NPK and ME) in mixotrophic cultivation untreated (UN) and pretreated (PR) sugarcane molasses.

except in NPK pretreated molasse that was a little higher with 650 kg^{-1} dry biomass compared with the others that were 520 kg^{-1} dry biomass (Table 3).

4. Discussion

The use of sugarcane molasses and alternatives culture media in the *H. pluvialis* growth parameters had effect on cell density and biochemical composition. Mixotrophic condition using sugarcane molasses, ME and NPK culture media were effective on microalgae biomass due to the influence and the availability of macronutrients and micronutrients, such as nitrogen and phosphorus. Biomass, chlorophyll-a, TIN and conductivity showed similar results regarding mixotrophic cultivation and culture media. Regarding to economic aspects ME culture medium was more advantageous, however the annual biomass production was reported as similar to all conditions and culture media except for NPK (650 kg^{-1} dry biomass) culture medium in pretreated molasses.

The flexibility of mixotrophic cultivation employs organic carbon and light as energy source, thus increasing microalgae growth (Subhash et al., 2017). Piasecka et al. (2017), reported that a maximum of 20 g L^{-1} of molasses as carbon source, seems to be appropriated, with quantities ranging from 2.3 to 20 g L^{-1}. In the present study the use of 0.75 g L^{-1} of sugarcane molasses as carbon source, promoted a cell density below 4 x 10^5 cell mL^{-1}.

The growth rate and the doubling time are related to the type of species and the availability of nutrients, as well as in the choice of a specific culture medium being able to

Table 3. Estimation of components cost (in US$ dollars)[a] and biomass production (kg^{-1} dry biomass) for NPK and ME cultures media producing 100,000 L of *Haematococcus pluvialis*.

Production Input	Culture media	
	NPK	ME
Cost US$		
Untreated molasses	103	103
Pretreated molasses	178	178
Cost of alternatives culture media[a]	847	357
Total annual costs (Untreated molasses)[a]	12,350	5,980
Total annual costs (Pretreated molasses)[a]	13,325	6,955
Biomass Production (kg^{-1} dry biomass)		
Yield of microalgae biomass untreated	40	40
Yield of microalgae biomass pretreated	50	40
Annual biomass production untreated	520	520
Annual biomass production pretreated	650	520

[a]Average exchange rate (November 2021) US$1.00 = R$ 5.67.

alter these rates (SundarRajan et al., 2019). *Haematococcus pluvialis* in NPK culture medium, presented better growth, protein and lipid contents, chlorophyll-*a*, cell density, TIN and TP concentrations, and conductivity than ME culture medium due to the high N, P and K concentration in the inorganic fertilizer. Sipaúba-Tavares et al. (2015) observed that *H. pluvialis* cultivation under alternative media (NPK and ME) and commercial medium (WC) in phototrophic condition (without molasses) had best results of growth rate, doubling time, cell density and chlorophyll-*a* in NPK than WC. In current study, low *H. pluvialis* growth in ME culture medium may be associated to the Mg concentrations that were three times higher than in NPK culture medium. In both culture media with pretreated molasses, the best results were reported mainly to NPK culture medium. According to Dong et al. (2020) and Ermis et al. (2020) Mg concentration above 1,600 µg may result in a slight reduction in cell concentration nonetheless, it has a specific position as the central element of the chlorophyll molecule and all microalgae species have absolute need for this element. The micro-element present in higher concentrations in ME culture medium was Fe (> 2 µg L^{-1}). It is important in some metabolic processes of microalgae. However, wasn't observed a direct relation of these microelements in the biochemical and biological conditions of *H. pluvialis*.

Protein (<50% biomass dry weight) and lipids content (<4.9% biomass dry weight) in both mixotrophic cultivation and culture media may have been observed due to the high concentration of total inorganic nitrogen mainly in the chemical composition of culture media with 105 mg L^{-1} for NPK and 95 mg L^{-1} for ME. The proteins contents appeared to be related to the nitrogen available in the medium. Li et al. (2019) demonstrated that the microalgae proteins were increased by elevating nitrogen concentrations, which induces stronger photo-protection mechanism in mixotrophic cultivation. However, lipid accumulation starts after nutrient step up for biomass growth, such as nitrogen starvation and not higher biomass productivity (Cheah et al., 2018).

The total annual biomass production of *H. pluvialis* was a little higher in pretreated molasses with NPK culture medium (650 kg^{-1} dry biomass) than the others with 520 kg^{-1} dry biomass. However, the annual cost of ME culture medium was two times lower than NPK culture medium. The results of this work confirm the efficiency in regards of biological data and production cost of *H. pluvialis* cultivation with the use of NPK and ME culture media. NPK culture medium compared with WC commercial culture medium usually used for *H. pluvialis* cultivation, registered 65.7% cost reduction for NPK culture medium due to the increase of dollar cost (Scardoeli-Truzzi and Sipaúba-Tavares, 2017). Compared to ME culture medium, the price advantage is even greater with a 77.5% cost reduction.

5. Conclusions

The NPK and ME culture media for *H. pluvalis* in mixotrophic cultivation promoted an annual biomass production being a bit higher in pretreated molasses in NPK culture medium. Maximum cell density, chlorophyll-*a*, nitrogen, protein and lipid contents were higher in NPK culture medium in pretreated sugarcane molasses, except cell carbon that was higher in the two mixotrophic cultivation in ME culture medium. The presence of nutrients in both culture media provided the conditions for employment in *H. pluvialis* cultivation. ME culture medium with aquatic plant *Eichhornia crassipes* presents an alternative that may be used as culture medium to *H. pluvialis*, due to the presence of macro and micro-nutrients, mainly Fe and Mg, that play critical role in a variety of metabolic pathways important for microalgae. This study reported the potential use of sugarcane untreated or pretreated molasses as a carbon source as well as the alternatives culture media as a good tool and new protocol for *H. pluvialis* cultivation.

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References

ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS – AOAC, 2012. *Official methods of analysis of AOAC International*. 19th ed. Gaithersburg: AOAC. Fatty acids in oils and fats. Preparation of methyl esters. Boron trifluoride method, p. 20.

CECCHIN, M., BENFATTO, S., GRIGGIO, F., MORI, A., CAZZANIGA, S., VITULO, N., DELLEDONNE, M. and BALLOTTARI, M., 2018. Molecular basis of autotrophic vs mixotrophic growth in *Chlorella sorokiniana*. *Scientific Reports*, vol. 8, no. 1, p. 6465. http://dx.doi.org/10.1038/s41598-018-24979-8. PMid:29691462.

CHEAH, W.Y., SHOW, P.L., JUAN, J.C., CHANG, J.S. and LING, T.C., 2018. Enhancing biomass and lipid productions of microalgae in palm oil mill effluent using carbon and nutrient supplementation. *Energy Conversion and Management*, vol. 164, pp. 188-197. http://dx.doi.org/10.1016/j.enconman.2018.02.094.

DONG, X., HUANG, L., LI, T., XU, J.W., ZHAO, P. and YU, X., 2020. The enhanced biomass and lipid accumulation in algae with an integrated treatment strategy by waste molasses and Mg^{2+}. *Energy Sources, Part A: Recovery, Utilization, and Environmental Effects*, vol. 42, no. 10, pp. 1183-1192. http://dx.doi.org/10.1080/15567036.2019.1602227.

ERMIS, H., GUVEN-GULHAN, U., CAKIR, T. and ALTINBAS, M., 2020. Effect of iron and magnesium addition on population dynamics and high value product of microalgae grown in anaerobic liquid digestate. *Scientific Reports*, vol. 10, no. 1, p. 3510. http://dx.doi.org/10.1038/s41598-020-60622-1. PMid:32103096.

GOLTERMAN, H.L., CLYMO, R.S. and OHNSTAD, M.A.M., 1978. *Methods for physical and chemical analysis of fresh water*. 2nd ed. Oxford: Blackwell Scientific Publications, 213 p. IBP Handbook, no. 8.

GUILLARD, R.R.L., 1973. Division rates. In: J.R. STEIN, ed. *Handbook of phycological methods: culture methods and growth measurements*. London: Cambridge University Press, pp. 289-311.

KADKHODAEI, S., ABBASILIASI, S., SHUN, T.J., FARD-MASOUMI, H.R., MOHAMED, M.S., MOVAHEDI, A., RAHIM, R. and ARIFF, A.B., 2015. Enhancement of protein production by microalgae *Dunaliella salina* under mixotrophic condition using response surface methodology. *RSC Advances*, vol. 5, no. 48, pp. 38141-38151. http://dx.doi.org/10.1039/C5RA04546K.

KOROLEFF, F., 1976. Determination of nutrients. In: E. GRASSHOFF and E. KREMLING, eds. *Methods of seawater analysis*. Weinheim: Verlag Chemie, pp. 126-133.

LAGE, S., KUDAHETTIGE, N.P., FERRO, L., MATSAKAS, L., FUNK, C., ROVA, U. and GENTILI, F.G., 2019. Microalgae cultivation for the biotransformation of birch wood hydrolysate and dairy effluent. *Catalysts*, vol. 9, no. 2, p. 150. http://dx.doi.org/10.3390/catal9020150.

LI, X., LI, W., ZHAI, J., WEI, H. and WANG, Q., 2019. Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. *Bioresource Technology*, vol. 273, pp. 368-376. http://dx.doi.org/10.1016/j.biortech.2018.11.042. PMid:30453251.

LIU, J., SUN, Z., ZHONG, Y., GERKEN, H., HUANG, J. and CHEN, F., 2013. Utilization of cane molasses towards cost-saving astaxanthin production by a *Chlorella zofingiensis* mutant. *Journal of Applied Phycology*, vol. 25, no. 5, pp. 1447-1456. http://dx.doi.org/10.1007/s10811-013-9974-x.

MONDAL, M., GHOSH, A., TIWARI, O.N., GAYEN, K., DAS, P., MANDAL, M.K. and HALDER, G., 2017. Influence of carbon sources and light intensity on biomass and lipid production of *Chlorella sorokiniana* BTA 9031 isolated from coalfield under various nutritional modes. *Energy Conversion and Management*, vol. 145, pp. 247-254. http://dx.doi.org/10.1016/j.enconman.2017.05.001.

NUSCH, E.A., 1980. Comparison of different methods for chlorophyll and phaeopigments determination. *Archiv für Hydrobiologie*, vol. 14, no. 1, pp. 14-36.

PIASECKA, A., KRZEMIŃSKA, I. and TYS, J., 2017. Enrichment of *Parachlorella kessleri* biomass with bioproducts: oil and protein by utilization of beet molasses. *Journal of Applied Phycology*, vol. 29, no. 4, pp. 1735-1743. http://dx.doi.org/10.1007/s10811-017-1081-y. PMid:28775655.

RATHER, Z.A. and NAZIR, R., 2015. Biochemical composition of selected macrophytes of Dal Lake, Kashimira Himalaya. *Journal of Ecosystem and Ecograph*, vol. 5, no. 1, pp. 4-36. http://dx.doi.org/10.4172/2157-7625.1000158.

SCARDOELI-TRUZZI, B. and SIPAÚBA-TAVARES, L.H., 2017. Sources of inorganic fertilizer in the growth of *Haematococcus pluvialis* Flotow (Chlorophyceae). *Journal of Algal Biomass Utilization*, vol. 8, no. 2, pp. 1-10.

SHAO, Y., GU, W., ZHU, Y., JIANG, L., ZHU, Y. and GONG, A., 2019. Study on the visualization of pigment in *Haematococcus pluvialis* by Raman spectroscopy technique. *Scientific Reports*, vol. 9, no. 1, p. 12097. http://dx.doi.org/10.1038/s41598-019-47208-2. PMid:31431631.

SIPAÚBA-TAVARES, L.H., BERCHIELLI-MORAIS, F.A. and SCARDOELI-TRUZZI, B., 2015. Growth of *Haematococcus pluvialis* Flotow in alternative media. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 75, no. 4, pp. 796-803. http://dx.doi.org/10.1590/1519-6984.23013. PMid:26675899.

SIPAÚBA-TAVARES, L.H., IBARRA, L.C. and FIORESI, T.B., 2009. Cultivo de *Ankistrodesmus gracilis* (REISCH) Korsikov (Chlorophyceae) em laboratório utilizando meio CHU_{12} e de macrófita com NPK. *Boletim do Instituto de Pesca*, vol. 35, no. 1, pp. 111-118.

SIPAÚBA-TAVARES, L.H., SCARDOELI-TRUZZI, B., FENERICK, D.C. and TEDESQUE, M.G., 2020. Comparison of photoautotrophic and mixotrophic cultivation of microalgae *Messastrum gracile* (Chlorophyceae) in alternative culture media. *Brazilian Journal of Biology = Revista Brasileira de Biologia*, vol. 80, no. 4, pp. 914-920. http://dx.doi.org/10.1590/1519-6984.226548. PMid:31800772.

SUBHASH, G.V., RAJVANSHI, M., KUMAR, B.N., GOVINDACHARY, S., PRASAD, V. and DASGUPTA, S., 2017. Carbon streaming in microalgae: extraction and analysis methods for high value compounds. *Bioresource Technology*, vol. 244, no. 2, pp. 1304-1316. http://dx.doi.org/10.1016/j.biortech.2017.07.024. PMid:28803061.

SUNDARRAJAN, P., GOPINATH, K.P., GREETHAM, D. and ANTONYSAMY, A.J., 2019. A review on cleaner production of biofuel feedstock from integrated CO_2 sequestration and wastewater treatment system. *Journal of Cleaner Production*, vol. 210, pp. 445-458. http://dx.doi.org/10.1016/j.jclepro.2018.11.010.

VOLLENWEIDER, R.A., 1974. *A manual on methods for measuring primary production in aquatic environments*. 2nd ed. Oxford: Blackwell Scientific Publications, 225 p. IBP Handbook, no. 12.

WANG, S.K., WANG, X., TAO, H.H., SUN, X.S. and TIAN, Y.T., 2018. Heterotrophic culture of *Chlorella pyrenoidosa* using sucrose as the sole carbon source by co-culture with immobilized yeast. *Bioresource Technology*, vol. 249, pp. 425-430. http://dx.doi.org/10.1016/j.biortech.2017.10.049. PMid:29065324.

WEN, X., TAO, H., PENG, X., WANG, Z., DING, Y., XU, Y., LIANG, L., DU, K., ZHANG, A., LIU, C., GENG, Y. and LI, Y., 2019. Sequential phototrophic-mixotrophic cultivation of oleaginous microalga *Grasiella* sp. WBG-1 in a 1000 m^2 open raceway pond. *Biotechnology for Biofuels*, vol. 12, no. 1, p. 27. http://dx.doi.org/10.1186/s13068-019-1367-1. PMid:30805027.

YEE, W., 2015. Feasibility of various carbon sources and plant materials in enhancing the growth and biomass productivity of the freshwater microalgae *Monoraphidium griffithii* NS16. *Bioresource Technology*, vol. 196, pp. 1-8. http://dx.doi.org/10.1016/j.biortech.2015.07.033. PMid:26210717.