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Application of Static Magnetic Fields on the Mixotrophic Culture of *Chlorella minutissima* for Carbohydrate Production

Bruno da Costa Menestrino[1] · Tamyris Helena Chaves Pintos[1] · Luisa Sala[1] · Jorge Alberto Vieira Costa[2] · Lucielen Oliveira Santos[1]

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Abstract
Magnetic field (MF) can interact with the metabolism of microalgae and has an effect (positive or negative) on the synthesis of molecules. In addition to MF, the use of pentose as a carbon source for cultivating microalgae is an alternative to increase carbohydrate yield. This study aimed at evaluating the MF application on the mixotrophic culture of *Chlorella minutissima* in order to produce carbohydrates. MF of 30 mT was generated by ferrite magnets and applied diurnally for 12 days. The addition of 5% pentose, MF application of 30 mT, and nitrogen concentration reduced (1.25 mM of KNO_3) was the best conditions to obtain higher carbohydrate concentrations. MF application of 30 mT increased biomass and carbohydrate contents in 30% and 163.1%, respectively, when compared with the assay without MF application. The carbohydrate produced can be used for bioethanol production.

Keywords Microalgae · Pentose · Magnetic fields · Chlorophyte · *Chlorella*

Introduction

Since energy moves society, environmental concerns and restrictions on the availability of land for energy production have led to the search for clean sources of high-yield biomass [1]. First-generation fuels used biomass. However, the feedstock (corn, sugar cane, and beet) is directly in competition with food production and other growing needs of today's society. The concept of second-generation (2G) biorefineries has been defined as the use of fermentable sugars

✉ Lucielen Oliveira Santos
santoslucielen@gmail.com

[1] Laboratory of Biotechnology, Chemistry and Food School, Federal University of Rio Grande, Rio Grande, RS 96203-900, Brazil

[2] Laboratory of Biochemical Engineering, Chemistry and Food School, Federal University of Rio Grande, Rio Grande, RS 96203-900, Brazil

extracted from the lignocellulosic portion of the sugarcane plant, such as bagasse to produce ethanol [2]. However, the yeast *Saccharomyces cerevisiae* employed in sugarcane biorefineries cannot ferment an important fraction (25%) of sugars available in the bagasse, i.e., pentose sugars. Although engineered microorganisms may be able to ferment pentoses, none of them have achieved high yield and fermentation productivity achieved by *S. cerevisiae* [3].

Microalgae biomass is an attractive source due macromolecules production, such as protein, carbohydrate, and lipid [4]. Carbohydrate and lipid need to be extracted from microalgae biomass for biofuels production. Changes in microalgae culture conditions may influence the synthesis or inhibition of these biomolecules. The use of pentose (xylose and/or arabinose) as a carbon source for microalgae cultivation is an alternative to increase ethanol yield [5–7]. There is also a decrease in the cost of the medium and it may stimulate carbohydrate accumulation in some strains that can be used for ethanol production. For example, Freitas et al. [8] evaluated the use of pentose (0.89 mg L^{-1} of arabinose and 19.16 mg L^{-1} of xylose), CO_2 (10 and 20%), and nitrogen content (2.5 and 1.25 mM) on *Chlorella minutissima* growth. The use of pentoses stimulated the carbohydrate production and inhibited the protein production in lower CO_2 and nitrogen concentration.

In addition, magnetic fields (MF) may interact with the metabolism and have an effect on the molecule synthesis. This treatment has advantages of being convenient, inexpensive, non-toxic, non-pollutant, widely applicable, and easily shielded [9]. MF responses are not always linear since they depend on the cell type, exposure time, frequency, and magnetic intensity [10]. Several studies have evaluated the effects of MF on microalga metabolism and cultivation and have observed an increase in biomass, carbohydrates, pigments, essential amino acids, and enzyme synthesis [9, 11–18]. In this study, we verified the interactions among MF application, addition of pentose, and reduction on nitrogen content during *Chlorella minutissima* cultivation in order to produce carbohydrates.

Material and Methods

Microorganism, Culture Medium, and Cultivation Conditions

The microalga *Chlorella minutissima* from the Collection of the Laboratory of Biochemical Engineering of the Federal University of Rio Grande (FURG), Rio Grande do Sul, Brazil, was used. Assays were performed with Modified Bristol's Medium (MBM) [19] which contains (g L^{-1}): 0.250/0.125 KNO_3; 0.01 $CaCl_2$; 0.075 $MgSO_4.7H_2O$; 0.075 K_2HPO_4; 0.175 KH_2PO_4; 0.025 NaCl; 0.02 $FeSO_4.7H_2O$; and 1 mL A5 solution. A5 solution was composed by (g L^{-1}): 2.86 H_3BO_3; 1.81 $MnCl_2.4H_2O$; 0.222 $ZnSO_4.7H_2O$; 0.079 $CuSO_4.5H_2O$; and 0.015 $NaMoO_4$.

The microalga was grown in 2 L vertical tubular photobioreactor (VTP) with 1.8 L of working volume. The initial biomass concentration was 0.3 g L^{-1}. Cultures were continuously aerated by the injection of sterile air (1260 mL min^{-1}). The cultivations were performed in a greenhouse with controlled temperature and photoperiod (model EL202/3, EletroLab, Brazil), and the conditions were photoperiod of 12 h light and 12 h dark, illuminance of 41 μmol m^{-2} s^{-1}, at 30 °C for 12 days [20]. Evaporation of water was controlled by daily addition of distilled water. Cultivations were performed in triplicate and control assay in duplicate.

C. minutissima cultivations are shown in Table 1. Three variables (nitrogen content, pentose addition, and MF application) were evaluated. In assays 2 cc, 2, 4 cc and 4 (Table 1), the medium was supplemented with 5% (v/v) pentose (D-xylose 19.16 mg L^{-1} and L-arabinose 0.89 mg L^{-1}), which represents the same amount of pretreated hydrolyzed sugarcane bagasse [8]. In the 3 cc, 3, 4 cc and 4 assays, a 50% reduction in nitrogen content (KNO_3 concentration) was evaluated. In cultures with MF application (assays 1, 2, 3, and 4, Table 1), ferrite magnets, with areas of 750 mm^2, were placed 180° from each other on the fixed walls of the VTP, 340 mm from the bottom. MF intensity was measured with a Globalmag MF measuring device (model TLMP-HALL-05 k-T0, Brazil) at several points/positions in the center of the VTP. Ferrite magnets generated a non-homogeneous (non-uniform) MF of 30 mT. The MF was applied continuously (24 h day^{-1}) for 12 days. In control cultivation (cc), the ferrite magnets were replaced by inert material to provide the same shading caused by the magnet.

At the end of the cultivation, the biomass was recovered from the liquid medium by centrifugation at 18400 ×*g*, at 20 °C for 30 min (model CR22GIII, HITACHI, Japan). The biomass was frozen for 48 h at − 80 °C and lyophilized by a lyophilizer (LABCONCO FreeZone6, USA). The lyophilized biomass was used to determination of protein and carbohydrate content.

Analytical Determinations

Biomass Concentration and pH

The biomass concentration (X, g L^{-1}) was assessed by measuring optical density of cultivations with a spectrophotometer (QUIMIB®, model Q798DP, Brazil) at 670 nm. The pH was measured by a digital pH meter Quimis (model Q400MT, Brazil). Both measurements were followed daily.

Evaluation of Growth Parameters

The maximum biomass concentration (X_{max}, g L^{-1}) was determined. The maximum productivity of biomass (P_{max}, g L^{-1} day^{-1}) was calculated by Eq. 1, where *X* represents

Table 1 Maximum biomass concentration (X_{max}, g L^{-1}); maximum productivity of biomass (P_{max}, g L^{-1} day^{-1}), maximum specific growth rate (μ_{max}, day^{-1}), and generation time (tg, day) for *Chlorella minutissima* cultivations with different concentrations of KNO_3, addition of pentose and magnetic fields (MF) application of 30 mT

Assay	KNO_3 (mM)	Pentose (%)	MF (mT)	X_{max} (g L^{-1})*	P_{max} (g L^{-1} day^{-1})*	μ_{max} (day^{-1})*	tg (day)*
1 cc**	2.50	0	0	0.50 ± 0.04^{d}	0.11 ± 0.06^{abcd}	0.097 ± 0.006^{d}	7.2 ± 0.4^{ab}
1	2.50	0	30	1.41 ± 0.17^{a}	0.13 ± 0.01^{ab}	0.232 ± 0.010^{a}	3.0 ± 0.1^{d}
2 cc**	2.50	5	0	0.55 ± 0.13^{d}	0.05 ± 0.01^{d}	0.148 ± 0.030^{c}	4.8 ± 1.0^{c}
2	2.50	5	30	1.35 ± 0.10^{a}	0.12 ± 0.02^{abc}	0.192 ± 0.013^{b}	3.6 ± 0.3^{cd}
3 cc**	1.25	0	0	0.89 ± 0.10^{bc}	0.15 ± 0.01^{a}	0.087 ± 0.003^{d}	8.0 ± 0.3^{a}
3	1.25	0	30	1.19 ± 0.07^{ab}	0.13 ± 0.02^{ab}	0.213 ± 0.006^{ab}	3.3 ± 0.1^{d}
4 cc**	1.25	5	0	0.70 ± 0.01^{cd}	$0.06 \pm < 0.01^{cd}$	0.102 ± 0.005^{d}	6.8 ± 0.3^{ab}
4	1.25	5	30	0.91 ± 0.01^{bc}	$0.07 \pm < 0.01^{bcd}$	0.112 ± 0.006^{cd}	6.2 ± 0.3^{b}

* Different letters indicate that there is significant difference between averages in the same column (Tukey's test, $p < 0.05$)

** *cc* control cultivation

the biomass concentration (g L^{-1}) at time t (day), and X_0 is the biomass concentration (g L^{-1}) on inoculation t_0 (day). The maximum specific growth rate (μ_{max}, day^{-1}) was obtained by exponential regression applied to the logarithmic phase growth. The generation time (tg, day) was calculated by Eq. 2, and represents the time (day) required for cell replication.

$$P_{max} = (X - X_0)/(t - t_0) \quad (1)$$

$$tg = \ln 2/\mu_{max} \quad (2)$$

Carbohydrate productivity (Y_{CHO}, g L^{-1} day^{-1}) was obtained by Eq. 3, where X_f represents the final biomass concentration (g L^{-1}), CHO is the carbohydrate concentration (%), and t is the cultivation time (day) [21].

$$Y_{CHO} = \left(X_f \times CHO\right)/(100 \times t) \quad (3)$$

The carbohydrate mass produced ($Mass_{CHO}$, g) was determined by Eq. 4, where X_f represents the final biomass concentration (g L^{-1}), CHO is the carbohydrate concentration (%), and V is the volume of cultivation (L).

$$Mass_{CHO} = (X_f \times CHO \times V)/100 \quad (4)$$

The effect of MF application on carbohydrate, protein, and biomass concentrations was evaluated and compared to their respective control assay (cc).

Determination of Carbohydrates and Proteins

The carbohydrate content was determined by the method described by DuBois et al. [22] whereas the protein content was determined by the method proposed by Lowry et al. [23], with an alkali-heat pre-treatment to obtain soluble protein.

Statistical Analysis

The responses X_{max}, protein, and carbohydrate contents were analyses by factorial ANOVA (analysis of variance) and followed by Tukey's test, considering a significance level of $p < 0.05$.

Results and Discussion

The choice of MF application with intensity of 30 mT on *Chlorella minutissima* cultivation was based on previously studies of Bauer et al. [17] and Deamici et al. [11], where different intensities (30 and 60 mT) and exposure time (1 h day^{-1} and 24 h day^{-1}) of application were evaluated on *Chlorella kessleri* and *Chlorella fusca*, respectively. In both studies, the highest carbohydrate concentrations (21.4% (w w^{-1}) for *C. kessleri* and 30.3% (w w^{-1}) for *C. fusca*) were obtained at 30 mT applied for 24 h day^{-1}.

Figure 1a shows biomass growth during 12 days of cultivation of *Chlorella minutissima*. MF application increased biomass growth when compared with its control cultures (cc),

differentiating from the 2-3th day of cultivation. In the control cultures (1 cc and 2 cc), the stationary phase started after the third day followed by death phase from the seventh day.

In the microalgae cultivation, the pH of the medium is an important factor that significantly affects growth. Changes in pH can affect enzymatic activities, solubility and availability of nutrients, transport of substrates across the cytoplasmic membrane, and the electron transport in photosynthesis and respiration [24]. The pH range 5–7 is indicated for growth of *C. minutissima* [25]. In all cultures, the initial pH was in the range of 6.4 to 7.2 and the pH value throughout the culture increased slightly, which in the end was in the range of 7.6 to 8.4 (Fig. 1b). The pH of assays with MF and without MF application had a similar behavior.

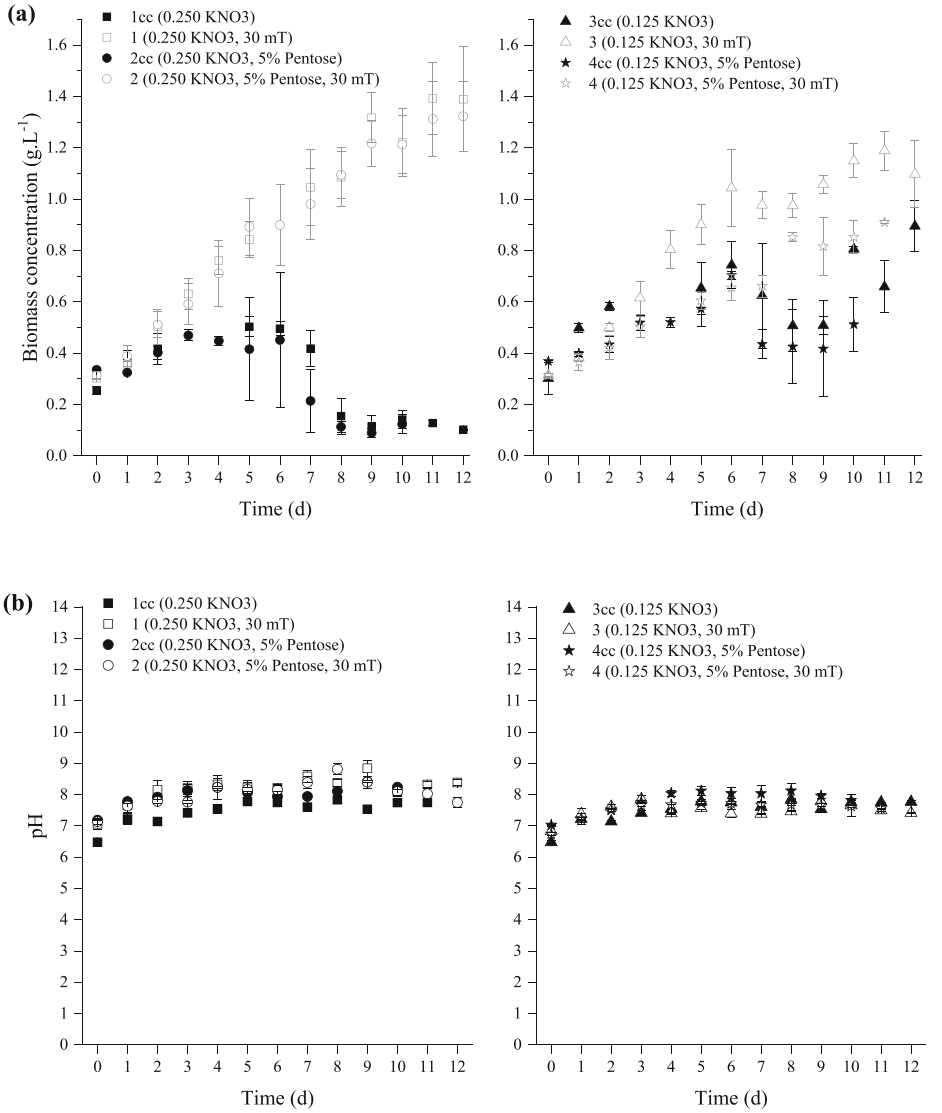

Fig. 1 (**a**) Biomass concentration and (**b**) pH during the cultivations of *Chlorella minutissima* with different concentrations of KNO_3, addition of 5% pentose, and magnetic fields application of 30 mT

Factorial ANOVA showed that pentose, MF, and the interactions between MF and KNO_3, pentose, and KNO_3 have a significant effect ($p < 0.05$) on the maximum biomass concentration (X_{max}). The pentose addition decreased X_{max}, while the application of 30 mT increased X_{max}, allowing an increase of 2.8, 2.4, 1.3, and 1.3 times in X_{max} for assays 1, 2, 3, and 4 (Table 1), respectively, by comparison with the control cultivations (cc). Therefore, MF of 30 mT can positively interact with the metabolism of microalgae *C. minutissima,* resulting in better kinetic parameters. The interactions between independent variables only have a positive effect on X_{max}, when the pentose was not added and KNO_3 was not reduced (Assay 1), or even when the MF was applied and KNO_3 was not reduced (Assays 1 and 2). The highest concentrations of X_{max} were 1.41, 1.35, and 1.19 g L^{-1} observed on Assays 1, 2, and 3 (Table 1), respectively.

The P_{max} and μ_{max} varied from 0.05 to 0.15 g L^{-1} day^{-1} and 0.087 to 0.232 day^{-1}, respectively. The highest μ_{max} (0.232, 0.213, and 0.192 day^{-1}) and the lowest generation time (3.0, 3.3, and 3.6 days) were found in the assays 1, 3, and 2, respectively, in which the highest values of X_{max} were found. The P_{max} and μ_{max} values for *C. minutissima* in literature range from 0.009–0.16 g L^{-1} day^{-1} and 0.008–0.74 day^{-1} [5, 7, 8, 21].

The factorial ANOVA showed that KNO_3, pentose, MF, and the interactions between KNO_3 and MF, pentose and MF, KNO_3 and pentose, and MF were significant effects ($p < 0.05$) on carbohydrate content. The addition of pentose and MF application has a positive effect on the carbohydrate content, while the increase in the KNO_3 concentration decreased the carbohydrate content in the microalgae. In addition, the interactions between independent variables showed that: (i) regardless of the MF application, the increase in KNO_3 concentration decreased the carbohydrate accumulation; (ii) regardless of whether or not pentose was added, MF application of 30 mT increased the carbohydrate accumulation; and (iii) MF application combined with pentose addition and regardless of the KNO_3 concentration promoted the carbohydrate accumulation (Assays 2 and 4, Table 2).

In agreement with our results, some researches have demonstrated that pentose addition on *C. minutissima* cultures reduced X_{max} and increased carbohydrate content [5, 7, 8], and KNO_3 reduction decreased the X_{max} [8]. Freitas et al. [7] verified that the pentose addition (1, 5, 10, 20, and 30%), with a 50% reduction in KNO_3 in the BMM medium, decreased X_{max} (0.39–0.51 g L^{-1}) by comparison with control cultivation (0.56 g L^{-1}). In addition, Freitas et al. [5]

Table 2 Content, productivity and mass of carbohydrate (CHO), and protein content at end of *C. minutissima* cultivations with different concentrations of KNO_3, addition of pentose, and magnetic field (MF) application of 30 mT

Assay	KNO_3 (mM)	Pentose (%)	MF (mT)	CHO content (% w w^{-1})**	CHO productivity (mg L^{-1} day^{-1})	CHO mass (g)	Protein content (% w w^{-1})**
1cc*	2.50	0	0	10.95 ± 0.25^{d}	$0.9 \pm < 0.1$	0.02 ± 0.00	5.21 ± 0.11^{c}
1	2.50	0	30	31.18 ± 1.23^{b}	33.3 ± 1.4	0.72 ± 0.04	12.84 ± 0.05^{b}
2cc*	2.50	5	0	14.40 ± 0.90^{cd}	1.8 ± 0.5	0.03 ± 0.01	5.04 ± 0.05^{c}
2	2.50	5	30	49.35 ± 5.24^{a}	56.8 ± 8.7	1.23 ± 0.03	12.41 ± 0.65^{b}
3cc*	1.25	0	0	32.43 ± 3.65^{b}	24.3 ± 3.8	0.53 ± 0.12	4.17 ± 2.69^{c}
3	1.25	0	30	28.07 ± 5.57^{bc}	25.4 ± 3.1	0.55 ± 0.08	3.48 ± 1.40^{c}
4cc*	1.25	5	0	22.99 ± 7.05^{bcd}	12.1 ± 4.3	0.22 ± 0.11	4.66 ± 1.16^{c}
4	1.25	5	30	60.48 ± 3.45^{a}	4.6 ± 8.2	0.99 ± 0.06	23.82 ± 0.91^{a}

* *cc* control cultivation

** Different letters indicate that there is significant difference between averages in the same column (Tukey's test, $p < 0.05$)

showed that the addition of 5% pentose increased the carbohydrate content (53.4%) compared with the control culture (39.2%). A decreased of 32% and 41% in X_{max} was noticed, when the culture was carried out with 50% reduction of KNO_3, and 10 and 20% of CO_2, respectively [8].

The addition of pentose and MF application had significant positive effect on protein content (factorial ANOVA, $p < 0.05$). The interactions KNO_3 and pentose, pentose and MF, and KNO_3 and pentose and MF showed significant effect on protein content (Table 2). The best conditions for protein accumulation by *C. minutissima* were MF application, pentose addition with reduced KNO_3 content (23.8%, Assay 4).

The addition of pentoses in *C. minutissima* cultivation did not increase the biomass concentration, but favored the intracellular macromolecules production, such as protein and carbohydrate. *C. minutissima* cultivations performed by Freitas et al. [8] with pentose (0.89 mg L^{-1} arabinose and 19.16 mg L^{-1} xylose), nitrogen content (1.25 and 2.5 mM) and CO_2 (10 and 20%) showed that addition of pentose only increased the carbohydrate content when 10% CO_2 and 1.25 mM KNO_3 were used, while the protein content was reduced and the biomass concentration has not been changed.

The 50% reduction of nitrogen (KNO_3) in *C. minutissima* cultivation affects only the carbohydrate content, being positive to carbohydrate accumulation. In agreement with Freitas et al. [6], for *C. minutissima*, the reduction in the nitrogen source in the medium did not change the biomass concentration. Therefore, the decrease in nitrogen concentration in the BMM medium was not restrictive in these conditions for the biomass production and increased the carbohydrate content.

Carbohydrate accumulation (% w w^{-1}) was observed in assays with a 50% reduction in KNO_3 (1.25 mM) (Table 2). The highest carbohydrate (60.5% w w^{-1}) and protein (23.8% w w^{-1}) contents were reached when KNO_3 was reduced 50% in the medium, pentose was added, and 30 mT was applied (assay 4, Table 2). This fact is in agreement with the results reported by Rigano et al. [26] and Freitas et al. [8], who reported that the reduction in the nitrogen source may increase the carbohydrate content in the genus *Chlorella*.

In our study, the MF application increased X_{max}, protein, and carbohydrate accumulation. Some studies have reported that the MF application on microalgae increased biomass [17, 13, 11, 15], macromolecules [17, 11, 16], and intracellular pigments [17, 15]. For example, Bauer et al. [17] showed that MF application (60 mT for 1 h day^{-1}) in *Chlorella kessleri* LEB 113 stimulated biomass, lipid, chlorophyll-*a*, chlorophyll-b, and carotenoid synthesis. Small et al. [13] observed that the application of 10 mT in the cultivation of *Chlorella kessleri* in a raceway reactor led to a doubling of the final biomass concentration, which increased from 0.88 g L^{-1} (control assay) to 1.56 g L^{-1}. Deamici et al. [11] showed that 60 mT applied for 24 h day^{-1} in *Chlorella fusca* LEB 111 increased the carbohydrate content by 24.8% and the biomass concentration by 20.5%. In addition, application of 30 and 60 mT (1 or 24 h day^{-1}) to *Spirulina* sp. LEB18 stimulated biomass concentration, protein (60 mT, 24 h day^{-1}) and carbohydrate (30 mT, 24 h day^{-1}) contents [16]. *Spirulina* sp. LEB18 biomass and chlorophyll-a content increased with 25 mT applied for 24 h day^{-1} [15].

MF causes oxidative stress in the cell [27, 28], affecting the metabolism cell. The interaction between MF and living cells is establish by three mechanisms: (i) magnetic induction, where the MF can exert forces on moving ions in solution, giving rise to induced electric fields and current; (ii) magneto-mechanical effect, the MF produce torques in certain molecules; and (iii) electronic interaction where the MF can change energy levels and spin orientation of

electrons [27]. These interactions between MF and cells alter the intracellular macromolecules production, increasing the protein and carbohydrate accumulation.

MF application increased the carbohydrate content in 184.7% (assay 1), 242.7% (assay 2), and 163.1% (assay 4) by comparison with their control cultures. Regarding the protein contents, the MF increases 146.4%, 146.2%, and 441.2%, for assays 1, 2, and 4, respectively. In all cultures, the carbohydrate mass produced with MF application was higher than that found in the control cultures. These events may have occurred because most metabolic reactions in living organisms depend on different load of molecules, such as metallic chemical elements (Fe and Mg), found in their constitution. They can generate changes in metabolism by MF application and increase the accumulation of biomolecules of interest.

Results with MF application, especially in assays 1, 2, and 4, showed that MF are able to increase the mass of carbohydrates produced by the microalga in all conditions under study. The carbohydrate accumulation (60.48% (w w^{-1})) occurs when MF was applied, 5% pentose was added, and nitrogen concentration was reduced (1.25 mM) in the BMM medium (Assay 4). This condition is favorable to carbohydrate production with a productivity of 0.046 g L^{-1} day^{-1} (Table 2) and a promising source for the ethanol production using microalgae, since most biomass is composed by carbohydrates.

Conclusion

The application of 30 mT for 24 h day^{-1} increased the biomass concentration, protein, and carbohydrate accumulation in *C. minutissima.* The highest intracellular carbohydrate concentration was 60.5% (w w^{-1}) when MF was applied with simultaneous addition of 5% pentose (xylose 19.16 mg L^{-1} and arabinoses 0.89 mg L^{-1}) and 1.25 mM nitrogen. These results show that the addition of 5% pentose as a carbon source and nitrogen reduction in the BMM medium with MF application is an alternative to farming since it allows to obtain higher carbohydrate levels.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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