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KINECTICS OF GROWTH AND pH FOR *Chlorella sorokiniana* CULTIVATED IN SUGARCANE VINASSE AND NPK FERTILIZER

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ABSTRACT

Microalgae have many biotechnological applications. However, reducing the cost of production in controlled environments is still a challenge. Thus, the aim of this work was to develop an alternative growth medium based on sugarcane (*Saccharum officinarum*) vinasse and the fertilizer NPK at different conditions to replace conventional defined media for *Chlorella sorokiniana.* Vinasse was (a) submitted to five clarification processes, (b) diluted in water (10, 25, 40, and 50%), (c) diluted in water (10, 25, 40, and 50%) and supplemented with 1% NPK. Water + 1% NPK was utilized as control. Cell density and pH were monitored for 35 days. *Chlorella sorokiniana* showed higher cell densities in the media containing vinasse at 25% (2.81 x 10^7 cells mL^{-1}), and 40% (2.75 x 10^7 cells mL^{-1}), both supplemented with 1% NPK (20:5:20), after 21 days of cultivation, which was more than five times higher than in clarified vinasse (maximum of 0.53 x10^7 cells mL^{-1} for filtered vinasse) at the same period. It was also higher than diluted vinasse without NPK supplementation (maximum of 1.63 x 10^7 cells mL^{-1} when 25% diluted). Vinasse diluted to 40% added to 1% of NPK stock solution (20:5:20) was found suitable for the cultivation of *C. sorokiniana*.

Keywords: Distillery residue. Clarification. Microalgae. Growth medium.

1 INTRODUCTION

Microalgae are photosynthetic microorganisms with different forms of carbon and energy metabolism: photoautotrophic, heterotrophic, and mixotrophic. The use of *Chlorella* sp. in biotechnological processes has numerous advantages due to their high growth rate, cell productivity, and CO_2 absorption, resistance to changes in the environment, ability to produce different biomolecules[1]. They are utilized for many purposes, including human and animal nutrition, biofuels, pharmaceuticals, and wastewater treatment, beyond others[2,3].

Chlorella sorokiniana presents rapid growth and biomass production, with the competitive advantage that it can be cultivated in mixotrophic environments[4]. Various organic carbon sources including glucose, glycerol, citric acid, methanol, and sodium acetate have been explored to maximize biomass production under mixotrophic cultivation conditions[5], but the costs of chemical reagents for creating the culture medium constitute one of the biggest problems in microalgae cultivation. The use of residual organic carbon sources may reduce the cost of microalgae cultivation, reusing excess nutrients found in industrial wastewater, which is beneficial to the environment. Inorganic NPK fertilizer is also an alternative culture medium to synthetic media. It is simple and widely available, dissolves easily, has a defined composition, with a high level of nitrogen and phosphorus that trigger a moderate pH in the medium[6].

Thus, the aim of this work was to develop an alternative growth medium based on sugarcane (*Saccharum officinarum*) vinasse and the fertilizer NPK at different conditions as a replacer of the conventional defined media, and to evaluate the development of *Chlorella sorokiniana.*

2 MATERIAL & METHODS

Clarification of sugarcane vinasse. The vinasse was obtained from a sugar and ethanol mills located in Dourados, MS, Brazil. The vinasse was clarified according to the methodology described elsewhere[7], outlined in Figure 1. It was diluted in 10, 25, 40 and 50% distilled water, 10, 25, 40 and 50% distilled water + 1% NPK.

Cultivation of Chlorella sorokiniana in clarified vinasse. The microalgae *Chlorella sorokiniana* (Trebouxiophyceae) was acquired from the André Tosello Foundation (Ref. 211-32; CTT 7727; IBVF 211-32, University of Seville, Spain). It was pre-cultivated in 250 mL Erlenmeyer flasks containing 100 mL of defined Sueoka medium in an orbital shaker incubator with constant stirring (200 rpm) at controlled temperature of 25 ± 2°C) and photoperiod (12 h light / 12 h dark) provided by fluorescent lamps with 2,500 lux for 7 days. The main cultivations were carried out in triplicate in 500 mL Erlenmeyer flask with 250 mL working volume and were started by centrifuging a certain amount of the pre-culture (18574 *g*, 5 min, 4 °C), resuspending cells in distillated water, and transferring a certain volume of the resuspended preculture to the main media, so that the initial cell concentration was 5.56 x 10^5 cells mL^{-1}. Cultivations were performed in an orbital shaker incubator at the same stirring, temperature, and photoperiod conditions described above for 35 days. Samples (3 mL) were collected every 7 days from the flasks to measure cell density using a Neubauer chamber count and the pH by potentiometric measurements.

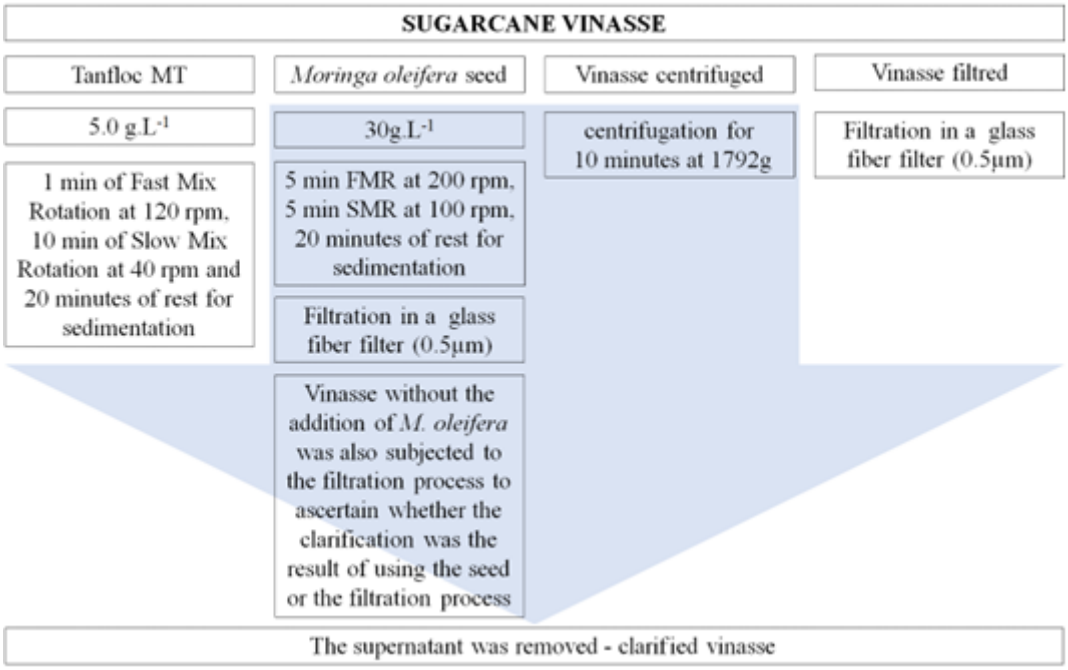

Figure 1 Procedures for vinasse clarification.

Statistical analysis. The evaluations were carried out from data obtained in triplicates, and the results were presented by the mean ± standard deviation. The software Genes version DOS (Visual Basic 5.0) was utilized to calculate the analysis of variance (ANOVA) and to determine the differences between treatments in the range of 95% confidence according to the Tukey test.

3 RESULTS & DISCUSSION

Results of cell density and pH of *C. sorokiniana* cultivated in different vinasse-based media for 35 days are presented in Tables 1 and 2, respectively.

Table 1. Cell density of *Chlorella sorokiniana* cultivated in different vinasse-based media for 35 days.

Medium	Cell density (x10^5 cell mL^{-1}) per time (days)					
	0	7	14	21	28	35
V1	$5.56±0.10^{NSde}$	$4.79±0.49^{Ie}$	$20.33±1.03^{Ha}$	$9.13±1.06^{Jc}$	$13.33±0.74^{Gb}$	$9.00±0.58^{HIJcd}$
V2	$5.56±0.10^{NSc}$	$4.67±0.37^{Ic}$	$15.79±0.58^{HIb}$	$14.19±0.11^{IJb}$	$19.19±0.18^{Ga}$	$5.00±0.58^{IJc}$
V3	$5.56±0.10^{NSe}$	$11.17±0.71^{Hd}$	$9.18±0.04^{Id}$	$21.94±0.95^{HIb}$	$42.15±1.03^{EFa}$	$15.58±0.65^{GHIc}$
V4	$5.56±0.10^{NSd}$	$24.71±01.12^{Gc}$	$38.42±0.55^{Gb}$	$52.17±1.16^{Ga}$	$51.81±1.08^{DEa}$	$48.63±0.87^{Fa}$
V5	$5.56±0.10^{NSe}$	$77.79±2.85^{Cc}$	$128.50±2.47^{Ca}$	$109±2.89^{Eb}$	$36.13±2.17^{Fd}$	$13.00±1.53^{GHIe}$
V6	$5.56±0.10^{NSf}$	$85.35±0.90^{Bc}$	$122±1.73^{Cb}$	$163.54±2.22^{Ca}$	$54.24±1.30^{Dd}$	$18.63±2.25^{GHe}$
V7	$5.56±0.10^{NSf}$	$76.23±0.72^{Cb}$	$109±2.89^{Da}$	$45.10±1.21^{Gc}$	$35.06±0.32^{Fd}$	$21.25±3.36^{Ge}$
V8	$5.56±0.10^{NSd}$	$66.94±1.46^{Da}$	$35.33±1.10^{GHb}$	$24.08±0.72^{Hc}$	$9.13±0^{Gd}$	$0J^{e}$
V9	$5.56±0.10^{NSe}$	$31.56±0.59^{Fd}$	$58.46±0.72^{Ec}$	$136.63±0.69^{Da}$	$61.79±0.63^{Db}$	$59.92±0.37^{Ebc}$
V10	$5.56±0.10^{NSf}$	$108.19±1.32^{Ae}$	$255.48±3.13^{Ab}$	$281.44±2.53^{Aa}$	$184.54±1.86^{Cc}$	$174.04±1.7^{Bd}$
V11	$5.56±0.10^{NSf}$	$75.85±1.11^{Ce}$	$224.40±2.26^{Bc}$	$275.15±0.20^{Aa}$	$253.60±2.06^{Ab}$	$210.63±0.95^{Ad}$
V12	$5.56±0.10^{NSf}$	$36.46±1.14^{Fe}$	$122±1.73^{Cd}$	$224.25±2.53^{Ba}$	$183.33±5.49^{Cb}$	$149.33±4.91^{Cc}$
V13	$5.56±0.10^{NSe}$	$48.17±1.09^{Ed}$	$48.25±0.45^{Fd}$	$69.10±2.83^{Fc}$	$208.05±4.62^{Ba}$	$112.07 ±4.04^{Db}$

V1) vinasse clarified with 30 g L^{-1} of *Moringa oleifera*; V2) vinasse clarified with 5 g L^{-1} of Tanfloc MT; V3) centrifuged vinasse (1792 *g* 10 min); V4) vinasse filtered through a glass fiber filter (0.5 µm); V5) 10% diluted vinasse V6) 25% diluted vinasse; V7) 40% diluted vinasse; V8) 50% diluted vinasse; V9) 10% diluted vinasse + 1% NPK; V10) 25% diluted vinasse + 1% NPK; V11) 40% diluted vinasse + 1% NPK; V12) 50% diluted vinasse + 1% NPK and a control (V13) prepared with distilled water and 1% NPK. *Averages followed by the same capital letter do not differ statistically from each other, comparing the cultivation media, in the column. Averages followed by the same lowercase letter do not differ statistically from each other, within each medium, between cultivation days, in the line, using the Tukey test at 5% probability.

Chlorella sorokiniana showed higher cell densities in the media containing vinasse at 25% (2.81 x 10^7 cells mL^{-1}), and 40% (2.75 x 10^7 cells mL^{-1}), both supplemented with NPK (20:5:20), after 21 days of cultivation, differing significantly from all the other culture media, including *C. sorokiniana* cultivated in clarified vinasse, (maximum of 0.53 x10^7 cells mL^{-1} for the vinasse clarified by filtering), and in diluted vinasse without NPK supplementation (maximum of 1.63 x 10^7 cells mL^{-1} when 25% diluted) at the same period (Table 1), reaching a fourfold difference over the control treatment.

Table 2. pH of *Chlorella sorokiniana* cultivated in different vinasse-based media for 35 days.

Medium	pH per time (days)					
	0	7	14	21	28	35
V1	5.07±0.05^{DEc}	7.02±0.58 ^{DEb}	9.39±0.13^{ABa}	9.31±0.03^{DEa}	9.30±0.03^{Ca}	9.62±0.01^{CDEa}
V2	5.02±0.04^{Ec}	7.38±0.76 ^{BCDEb}	8.83±0.04^{DEab}	9.08±0.01^{EFa}	8.79±0^{DEab}	9.31±0.01^{DEFa}
V3	5.16±0.03^{DEd}	7.22±0.25 ^{CDEc}	8.93±0.05^{CDb}	9.38±0.01^{CDEab}	9.31±0.04^{Cab}	9.47±0.00^{CDEa}
V4	5.10±0.02^{DEe}	6.97±0.20 ^{Ed}	8.75±0.06^{DEFc}	9.24±0.03^{DEFb}	9.28±0.02^{Cb}	9.76±0.03^{BCDa}
V5	5.53±0.05^{CDEc}	8.45±0.06 ^{ABCDEb}	8.79±0.10^{DEFab}	9.08±0.08^{EFa}	8.84±0.13 ^{DEab}	9.08±0.01^{EFa}
V6	5.21±0.01^{DEd}	8.16±0.03 ^{ABCDEc}	8.59±0.04^{EFb}	8.81±0.05^{FGa}	8.76±0.04^{Eab}	8.79±0.17^{Fab}
V7	5.08±0.01^{DEc}	8.28±0.01^{ABCDEb}	8.20±0.04^{Gb}	8.61±0.10^{Ga}	8.80±0.02^{DEa}	8.80±0.05^{Fa}
V8	5.01±0.00^{Ef}	8.17±0.01^{ABCDEe}	8.49±0.01^{FGd}	8.99±0.01^{EFGb}	8.81±0.02^{DEc}	9.24±0.03^{DEFa}
V9	6.57±0.13^{Bd}	9.03±0^{Ac}	9.18±0.02^{BCc}	9.65±0.02^{BCDb}	9.54±0.07^{BCb}	10.70±0.00^{Aa}
V10	6.06±0.01^{BCe}	8.86±0.05^{ABd}	9.41±0.04^{ABc}	9.99±0.01^{ABb}	10.57±0.06^{Aa}	10.71±0.08^{Aa}
V11	5.60±0.01^{CDEd}	8.74±0.03^{ABCc}	9.66±0.01^{Ab}	10.32±0.06^{Aa}	10.28±0.02^{Aa}	10.33±0.08^{ABa}
V12	5.65±0.07^{CDc}	8.55±0.01^{ABCDb}	9.34±0.10^{Bab}	9.78±0.27^{BCa}	9.82±0.26^{Ba}	9.86±0.027^{BCa}
V13	8.93±0.39 ^{Ans}	8.32±0.44^{ABCDEns}	9.31±0.04^{Bns}	9.26±0.05^{DEns}	9.21±0.05^{CDns}	9.50±0.05^{CDEns}

Media composition according to Table 1. *Averages followed by the same capital letter do not differ statistically from each other, comparing the cultivation media, in the column. Averages followed by the same lowercase letter do not differ statistically from each other, within each medium, between cultivation days, in the line, using the Tukey test at 5% probability.

This inhibition in growth up to up to five times compared to the media prepared with clarified vinasse indicates that the high concentration of some elements may have exceeded the adaptive capacity of microalgae cells in some fundamental aspect related to their microalgae metabolism[8]. Vinasse, even if treated, has more nutrients in its composition than the commercial media utilized, including some metals that may be toxic to cells. In addition, the high color and turbidity, especially in the undiluted vinasse, may have interfered in the photosynthesis, limiting cell growth. Thus, a combination of factors may have influenced microalgae metabolism.

Literature reports that most of the microalgae species and strains do not successfully grow well in vinasse. In a study with 40 microalgae strains evaluated on undiluted vinasse under non-axenic conditions, and only the strains *Micractinium* sp. Embrapa-LBA32 and *C. biconvexa* Embrapa-LBA40 were selected due to their vigorous growth[9]. Thus, each microalgal strain needs to be evaluated individually. Here, the use of diluted sugarcane vinasse added of 1% NPK resulted in high production of microalgal biomass (Table 1).

The pH of the culture medium varied between 5.02 and 10.71, after the initial treatment and during sampling of the different media (Table 2). It observed a significant difference between the treatments, except for the control. The optimal pH for microalgae growth varies for each species, but in general it is close to neutrality. At this pH range, theoretically little energy is needed to maintain homeostasis[10], which is extremely important in microalgae cultivation. pH determines the solubility of carbon dioxide and minerals in the medium and directly or indirectly influences microalgae metabolism. *C. sorokiniana* cultivated in vinasse with NPK showed higher microalgal density at alkaline pH. The instability of the medium in maintaining the pH shows that these variations were possibly the result of the vinasse composition itself, even for the media with diluted vinasse, because this behavior was not observed for the control (water with 1% NPK).

4 CONCLUSION

Diluted vinasse proved to be a suitable medium for the microalgal growth of *C. sorokiniana*. The inhibition of the growth of *C. sorokiniana* in clarified vinasse was possibly due to the high composition of chemical elements that may have affected the metabolism the strain, inhibiting growth, and the high color and turbidity of vinasse, inhibiting photosynthesis, and consequently growth.Thus, to mitigate the environmental impacts of vinasse, it can be used diluted to 40% and added to 1% of NPK stock solution (20:5:20) for the cultivation of C*. sorokiniana,* presenting cell density four times greater than in the control medium.

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