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PRODUCTION OF BIODIESEL FROM MICROALGAE CULTIVATED WITH VINASSE

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ABSTRACT

Purpose: The objective of this work was to carry out the cultivation of the microalgae Chlorella sorokiniana with diluted vinasse, verifying its potential for remediation of nutrients and increase of its lipid fraction, to produce biodiesel according to the current regulation.

Theoretical Framework: The quest to reduce fossil fuels, which are responsible for negative environmental impacts, has boosted the demand for biofuels in recent years. Biodiesel has been gaining prominence as a promising alternative to replace petroleum derivatives since it can be produced from various raw materials, such as microalgae.

Design/Methodology/Approach: Different vinasse dilutions were studied for the cultivation of Chlorella sorokiniana CTT 7727, being 5, 10 and 15% (v v-1) and N:P:K (20:5:20) 1% (v v-1) medium as a comparative standard. Biodiesel was produced by direct transesterification and analyzed by gas chromatography coupled to a flame ionization detector, enabling the identification and quantification of the fatty acid profile.

Findings: The results obtained indicated that vinasse 10% (v v-1) proved to be promising for application as a culture medium for this microalgae species, as it guaranteed a good cell growth rate, high biomass productivity and lipid yield. Considering the biodiesel produced, it fits the regulatory standards analyzed by the National Agency of Petroleum, Natural Gas and Biofuels (ANP).

Research, Practical & Social Implications: The research demonstrates the feasibility of cultivating the microalga Chlorella sorokiniana with diluted vinasse, highlighting the 10% concentration as promising for optimizing cell growth and lipid yield. The results ensure compliance with ANP regulations for biodiesel production, indicating immediate and sustainable applicability on an industrial scale. Socially, it contributes to the pursuit of biofuels, reducing dependence on fossil sources and promoting more sustainable practices in the bioenergy industry.

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Originality/Value: The study's originality lies in the unique combination of Chlorella sorokiniana CTT 7727, diluted vinasse, and biodiesel production in accordance with ANP regulations. It stands out for identifying the optimal vinasse concentration (10%) for cultivation, presenting an economical and effective approach. The strategic value is in the use of an alternative raw material cultivated with an industrial residue, promoting the diversification of the energy matrix and mitigating environmental impacts associated with conventional fuels.

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PRODUÇÃO DE BIODIESEL DE MICROALGAS CULTIVADAS COM VINHAÇA

RESUMO

Objetivo: O objetivo deste trabalho foi realizar o cultivo da microalga *Chlorella sorokiniana* com vinhaça diluída, verificando seu potencial de remediação de nutrientes e incremento de sua fração lipídica, para produzir um biodiesel segundo a regulamentação vigente.

Referencial Teórico: A busca pela diminuição de combustíveis fósseis, responsáveis por impactos ambientais negativos impulsionou a demanda por biocombustíveis nos últimos anos. O biodiesel vem ganhando destaque como alternativa promissora em substituição aos derivados do petróleo, o mesmo pode ser produzido por diversas matérias-primas, como as microalgas.

Desenho/Metodologia/Abordagem: Estudaram-se diferentes diluições de vinhaça para o cultivo de *Chlorella sorokiniana* CTT 7727, sendo 5, 10 e 15% (v v-1) e N:P:K (20:5:20) 1% (v v-1) como padrão comparativo. O biodiesel foi produzido por transesterificação direta e analisado por cromatografia gasosa acoplada a detector de ionização de chama, possibilitando a identificação e quantificação do perfil de ácidos graxos.

Resultados: Os resultados obtidos indicaram que a vinhaça 10% (v v-1) mostrou-se promissora para aplicação como meio de cultivo desta espécie de microalga, pois garantiu uma boa taxa de crescimento celular, elevada produtividade de biomassa e rendimento lipídico. Considerando o biodiesel produzido, o mesmo enquadra-se nos padrões de regulamentação analisados dispostos pela Agência Nacional de Petróleo, Gás Natural e Biocombustíveis (ANP).

Pesquisa, Implicações Práticas e Sociais: A pesquisa evidencia a viabilidade do cultivo da microalga *Chlorella sorokiniana* com vinhaça diluída, destacando a concentração de 10% como promissora para otimização do crescimento celular e rendimento lipídico. Os resultados asseguram conformidade com as regulamentações da ANP para produção de biodiesel, indicando aplicabilidade imediata e sustentável em escala industrial. Socialmente, contribui para a busca por biocombustíveis, reduzindo a dependência de fontes fósseis e promovendo práticas mais sustentáveis na indústria bioenergética.

Originalidade/Valor: A originalidade do estudo reside na combinação única da Chlorella sorokiniana CTT 7727, vinhaça diluída e produção de biodiesel em conformidade com normativas da ANP. Destaca-se pela identificação da concentração ideal de vinhaça (10%) para cultivo, apresentando uma abordagem econômica e eficaz. O valor estratégico está na utilização de uma matéria-prima alternativa cultivada com um resíduo industrial, promovendo a diversificação da matriz energética e mitigando impactos ambientais associados aos combustíveis convencionais.

Palavras-chave: Biocombustível, *Chlorella sorokiniana*, Extração de Lipídios, Efluentes, Remoção de Nutrientes.

PRODUCCIÓN DE BIODIÉSEL A PARTIR DE MICROALGAS CULTIVADAS CON VINAZA

RESUMEN

Objetivo: El propósito de esta investigación fue llevar a cabo el cultivo de la microalga Chlorella sorokiniana con vinaza diluida, evaluando su potencial para la remediación de nutrientes y el aumento de su fracción lipídica, con el fin de producir biodiesel de acuerdo con la normativa vigente.

Marco Teórico: La búsqueda de la reducción de los combustibles fósiles, responsables de impactos ambientales negativos, ha impulsado la demanda de biocombustibles en los últimos años. El biodiesel ha ganado relevancia como una alternativa prometedora para reemplazar los derivados del petróleo, pudiendo ser producido a partir de diversas materias primas, como las microalgas.

Diseño/Metodología/Enfoque: Se estudiaron diferentes diluciones de vinaza para el cultivo de Chlorella sorokiniana CTT 7727, siendo estas del 5%, 10% y 15% (v/v), con N:P:K (20:5:20) al 1% (v/v) como estándar comparativo. El biodiesel se produjo mediante transesterificación directa y se analizó mediante cromatografía de gas acoplada a detector de ionización de llama, permitiendo la identificación y cuantificación del perfil de ácidos grasos.

Resultados: Los resultados obtenidos indicaron que la vinaza al 10% (v/v) se mostró prometedora para su aplicación como medio de cultivo de esta especie de microalga, asegurando una buena tasa de crecimiento celular, una elevada productividad de biomasa y un rendimiento lipídico notable. En cuanto al biodiesel producido, este cumplió con los estándares de regulación establecidos por la Agencia Nacional de Petróleo, Gas Natural y Biocombustibles (ANP).
Investigación, Implicaciones Prácticas y Sociales: La investigación destaca la viabilidad del cultivo de la microalga Chlorella sorokiniana con vinaza diluida, señalando la concentración del 10% como promisoria para optimizar el crecimiento celular y el rendimiento lipídico. Los resultados aseguran la conformidad con las regulaciones de la ANP para la producción de biodiesel, indicando una aplicabilidad inmediata y sostenible a nivel industrial. Desde el punto de vista social, contribuye a la búsqueda de biocombustibles, reduciendo la dependencia de fuentes fósiles y promoviendo prácticas más sostenibles en la industria bioenergética.
Originalidad/Valor: La originalidad del estudio radica en la combinación única de *Chlorella sorokiniana* CTT 7727, vinaza diluida y producción de biodiesel en conformidad con las normativas de la ANP. Se destaca por identificar la concentración ideal de vinaza (10%) para el cultivo, presentando un enfoque económico y eficaz. El valor estratégico reside en el uso de una materia prima alternativa cultivada con un residuo industrial, fomentando la diversificación de la matriz energética y mitigando los impactos ambientales asociados a los combustibles convencionales.

Palabras clave: Biocombustible, *Chlorella sorokiniana*, Extracción de Lípidos, Efluentes, Remoción de Nutrientes.

1 INTRODUCTION

The increase in the concentration of pollutants dispersed in the atmosphere and the decreasing availability of fossil fuels reinforce the need to look for less polluting fuels from renewable sources. From this perspective, biodiesel has emerged as an alternative to oil and its derivatives, as it can be produced from various renewable raw materials, and because it emits fewer pollutants when considering its production and consumption cycle (Nassef et al., 2019).

Studies have identified microalgae as an excellent alternative for biodiesel production Hyppolito et al. (2021) and Wang et al. (2019). Among the main characteristics of microalgae, Chisti (2007) we highlight their considerable lipid content, their ability to double their biomass in a short space of time and the smaller space they take up for cultivation compared to other oilseeds used for this purpose.

Cultivating these organisms in the laboratory requires the use of suitable nutrient sources, such as alternative sources like vinasse. Vinasse is an industrial effluent known for its considerable concentrations of macro and micronutrients, which are essential for the development of plant species such as microalgae (Candido et al., 2021).

To produce biodiesel, lipid extraction from microalgae is necessary. In his study, Oshita and Takaoka (2021) classified ethanol as the best solvent for extracting oil from microalgae, also stating that by reducing the volume of ethanol by half, its extraction potential is not altered, which makes it economically and environmentally viable.

After extracting the microalgal lipids, the transesterification reaction process is carried out. Mathew et al. (2021) in transesterification, the triglyceride reacts with an alcohol to produce ester and glycerin. To make this possible, a catalyst is added to the reaction. The overall process is usually a sequence of three consecutive steps, which are reversible reactions. In the first step, diglycerides are obtained from triglycerides, monoglycerides are produced from diglycerides and the last step from monoglycerides, crude glycerine.

This work used vinasse as a medium for growing microalgae to obtain oil for biodiesel production. To this end, the rates of nutrient removal from vinasse during cultivation were studied, as well as the best solvents for extracting oil from the microalgae biomass, aiming for good yields and an environmentally suitable and clean process.

2 MATERIALS AND METHODS

The microalgae species used for cultivation was Chlorella sorokiniana CTT 7727. The microalgae were cultivated for a period of 30 days in Erlenmeyer flasks using diluted vinasse as a growing medium, with concentrations equivalent to 5, 10 and 15% (v v-1). The chemical fertilizer N:P:K (20:5:20) at a concentration of 1% (v v-1) was used as a standard for comparison. All treatments were carried out in triplicate.

The tests were carried out in a non-axenic environment, with constant aeration from a Minjiang model PS-950 electronic blower, with an air flow equivalent to 125 L h-1 for each sample, an average temperature of 25.6ºC and a 12/12h light/dark photoperiod with a light intensity equivalent to 2400 lux. Table 1 shows the composition of the samples for each test.

Table 1

Composition of Chlorella sorokiniana cultivation samples.

Treatment (v v^{-1})	Concentration (%)	Growing medium (mL)	Water (mL)	Harmless (mL)	Volume (mL)
Vinasse	5	90	1710	200	2000
	10	180	1620	200	2000
	15	270	1530	200	2000
N:P:K (20:5:20)	1	18	1782	200	2000

Dilutions were made in relation to a volume of 1800 mL. The cell multiplication of the microalgae during cultivation was determined from the optical density (OD) and dry microalgae biomass.

The absorbance was analyzed at a wavelength of 680 nm every 5 days until the end of cultivation, using a UV/Visible spectrophotometer (Rayleigh, model UV-9200). The maximum specific growth (μmax) was determined from the slope of the line and the doubling time (TD) was determined from the ratio between ln(2)/μmax.

At the same time, the increase in dry biomass during cultivation was assessed, and samples were taken from each replicate of the different treatments every 5 days. After obtaining the results, the linear correlation between absorbance and dry microalgal biomass was verified using Pearson's test.

At the end of cultivation, the cellular composition was analyzed, quantifying the protein, carbohydrate and ash contents (AOAC, 2005). The micro-Kjeldahl method was used to determine protein, with a correction factor of 6.25, ash by burning in a muffle furnace at a temperature of 800ºC and carbohydrate content by means of the difference in centesimal composition. The lipid content was determined using the Bligh and Dyer method, adapted by D'Oca et al. (2011)

2.1 NUTRIENT REMOVAL DURING CULTIVATION

The removal of nutrients from vinasse during cultivation was carried out by means of physico-chemical analysis, determining the concentration of ammonia, nitrite, nitrate and phosphate at the beginning and end of the cultivation period. Quantification was based on Equation 1 (appendix).

2.2 BIOMASS RECOVERY

The biomass of Chlorella sorokiniana was recovered from the cultivation medium using a set of unit operations consisting of coagulation/flocculation, decantation, filtration and drying. For coagulation/flocculation, ferric chloride hexahydrate at a concentration of 0.75 g L-1 was used as the flocculating agent (Menegazzo et al., 2021). After the flocculation procedure, the samples were left to stand for 24 hours to decant the microalgae cells. The supernatant was drained and the fraction containing the decanted cells was filtered through a fabric filter. The cells contained in the filter were placed in weighed porcelain capsules and sent to the oven at a temperature of 60ºC until they reached a constant mass. The capsules were removed from the oven and cooled in a desiccator, where they remained for 30 minutes. They were then weighed

on an analytical balance and the dry biomass was obtained. After drying, the biomass was stored at -4 ºC until use.

2.3 LIPID EXTRACTION

The methodology proposed by Bligh and Dyer adapted by D'Oca et al. (2011) was used to extract the lipids. Different solvents were analyzed in order to determine which would provide the greatest amount of oil extracted per mass of dried microalgae: a 2:1 (v v-1) chloroform-methanol mixture, methanol, ethanol and hexane.

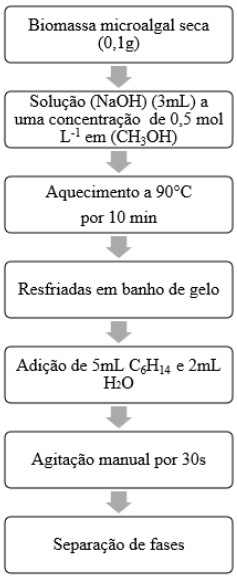
The dried biomass was added to the chemical solvent under magnetic stirring at 400 rpm or in an ultrasound bath (42 kHz frequency) for a period of 30 minutes. The samples were then centrifuged at 2000 rpm for 5 min. Finally, the organic fraction was collected and taken to an oven at 60ºC to evaporate the solvent. The resulting biomass in the test tube was submitted to the re-extraction process.

2.4 BIODIESEL PRODUCTION AND FATTY ACID PROFILE ANALYSIS

Biodiesel was produced by a direct organic transesterification reaction, as shown in Figure 1.

Figure 1

Flowchart of the biodiesel and fatty acid production process.



After the phases were formed, the upper phase, consisting of the fatty acids and hexane, was transferred to a vial GC and sent for analysis in gas chromatography coupled to a flame ionization detector. A ZebronTMZB-FFAP polar capillary column (30m x 0.32 mm 0.25 μm film thickness; Phenomenex, Torrance, CA, USA) was used to separate the fatty acids, with helium as the carrier gas.

The compounds present in the samples were identified and quantified by comparing the retention times and peaks with the standards established by Marine Oil Test Mix and FAME (Restek Corp., Bellefonte, PA, USA). 13The mass fractions were normalized as a percentage of the total fatty acids.

Chromatographic analysis was used to determine the characteristics of the fatty acids present in the biodiesel, such as degree of instauration (DI), cetane number (CN), iodine index (II), saponification index (SI), cloud point (CP), cold filter plugging point (CFPP), calorific value (CV), kinematic viscosity (v) and density (ρ). The empirical equations used were according to the methodology of Talebi et al. (2014); Ramírez-Verduzco et al. (2012) and Ramos et al. (2009) are attached.

2.5 STATISTICAL ANALYSIS

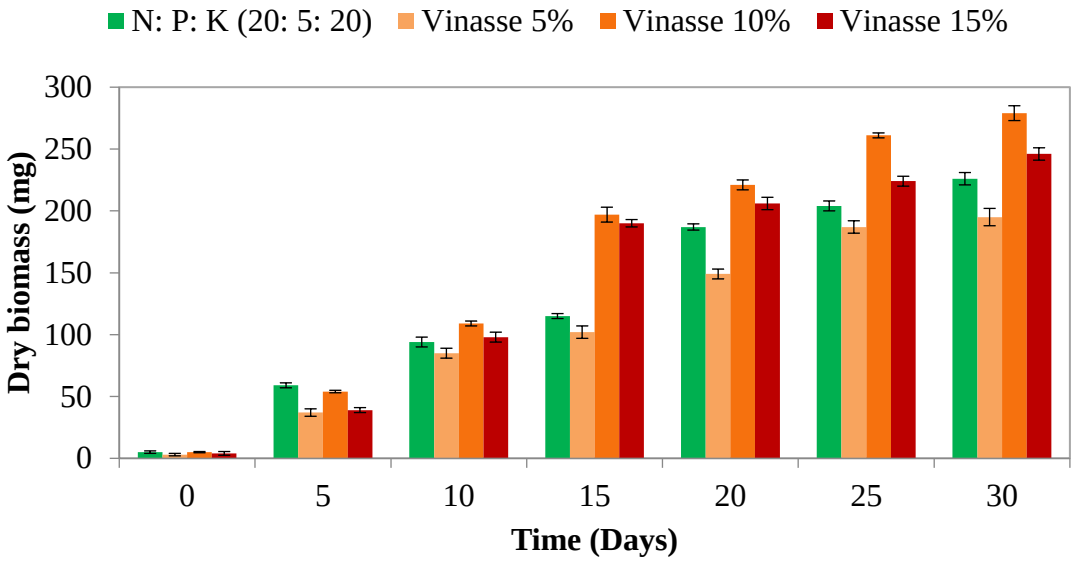
The data obtained from all the experiments was subjected to analysis of variance (ANOVA) using Minitab 19® software.17

3 RESULTS AND DISCUSSIONS

The adaptation of Chlorella sorokiniana to vinasse and, consequently, its cell growth, were monitored throughout the cultivation period by analyzing the amount of dry biomass every 5 days, as shown in Figure 2.

Figure 2

Increase in dry biomass over the cultivation period.



In Figure 2, the cultures submitted to cultivation with diluted vinasse showed good adaptation to the growing medium and a positive increase in dry biomass at the end of the experiment, with the 10% (v v-1) vinasse treatment standing out, which guaranteed the best results in the study. Ansilago et al. (2021) also noted the evolution of cell growth and the increase in dry biomass of Chlorella sorokiniana cultivated with 1% (v v-1) vinasse in a non-axenic medium, indicating that the medium in question has potential for application in the cultivation of microalgae of this species.

Ramirez et al. (2014) analyzed the cultivation of the microalga Scenedesmus sp. with vinasse at dilutions of 12.5, 25.0, 37.5 and 50.0% and identified an increase in biomass at all the concentrations tested, although the higher concentrations showed a lower biomass growth compared to the lower concentrations, highlighting that cultivations with this effluent should be carried out at a volume of up to 40% (v v-1).

In addition to the increase in dry biomass, the variation in absorbance was also monitored over the days of cultivation for all the tests with vinasse and for the control, as shown in Figure 3.

Figure 3

Growth curves of Chlorella sorokiniana cultures with N:P:K (20:5:20) and Vinasse 5, 10 and 15% (v v-1).

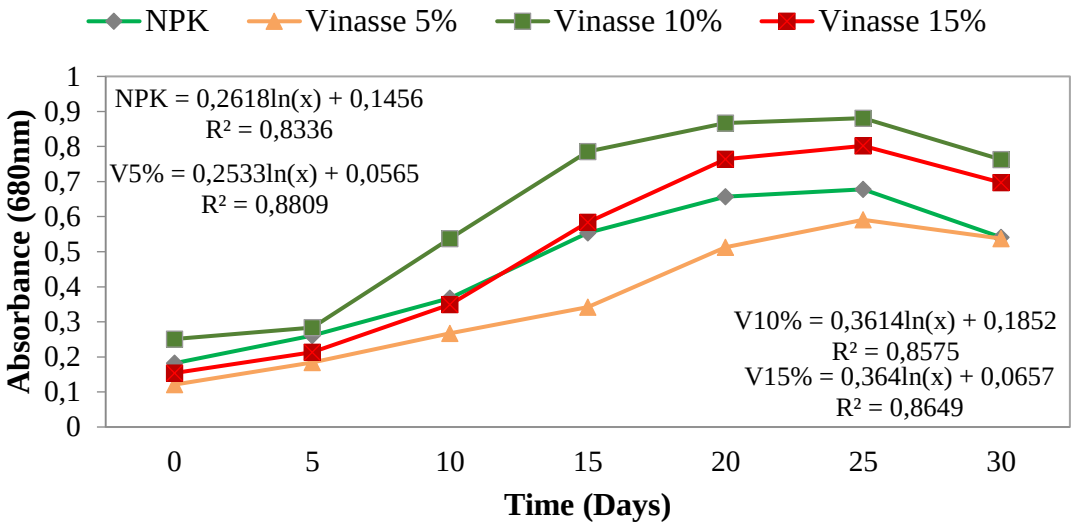


Figure 3 shows the growth profile of Chlorella sorokiniana according to the variations in absorbance every 5 days of cultivation. It can be seen that, with the exception of the control, all the tests showed growth curves with 4 distinct phases, starting with the induction phase (0 to 5 days), followed by the exponential phase (5 to 20 days), passing through the stationary phase (20 to 25 days) and, finally, the decline phase (25 to 30 days). In order to confirm the correlation between the increase in absorbance over the course of the experiment and the increase in dry biomass, a diagram was drawn up and Pearson's test carried out, as shown in Figure 4.

Figure 4

Correlation between dry biomass and absorbance at 680 nm for the different treatments with vinasse (v v-1) and N: P: K (20: 5: 20).

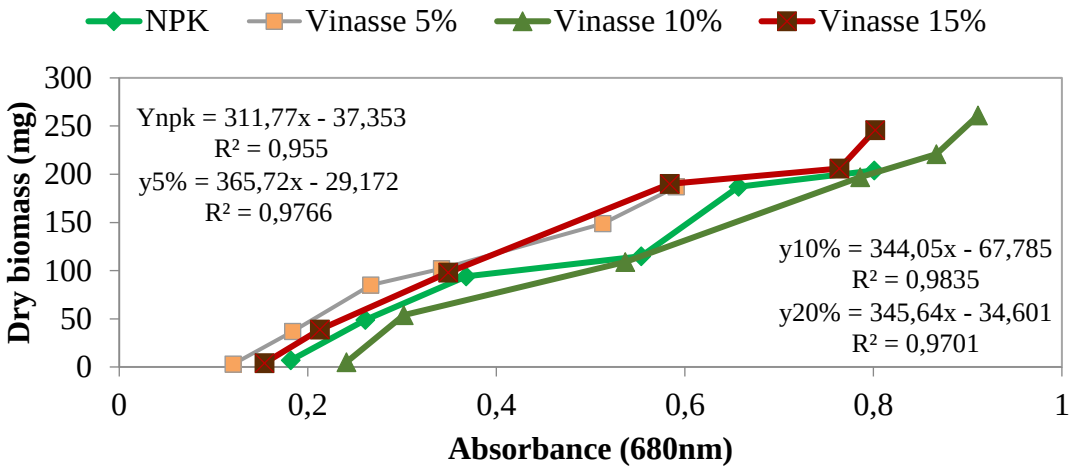


Figure 4 shows that dry biomass increases in line with the increase in absorbance at 680 nm, a fact confirmed by the Pearson test, which found correlation coefficients of 0.98, 0.99, 0.99 and 0.98 for the tests with the control and vinasse at dilutions of 5, 10 and 15%, respectively. Pearson's test indicates the linear association between two variables, this association is represented by the linear correlation coefficient (R), as20 the closer it is to 1, the greater the correlation between the variables studied, so it can be said that dry biomass increases proportionally to the increase in absorbance at 680 nm.

Based on the data shown in Figure 4, we also determined the maximum specific growth, doubling time and biomass productivity for each treatment studied, all the results of which are shown in Table 2.

Table 2

Growth and productivity of Chlorella sorokiniana under the conditions studied.

Treatment	μ_{max} (Day $^{-1}$)	Doubling time (Day)	Biomass productivity (mg L^{-1} Dia^{-1})
N:P:K(20:5:20) 1%	0,26 ± 0,03	2,65 ± 0,04	150,67 ± 1,02
Vinasse 5% (v v^{-1})	0,25 ± 0,02	2,74 ± 0,03	136,67 ± 2,08
Vinasse 10% (v v^{-1})	0,36 ± 0,01	1,92 ± 0,02	186,01 ± 1,79
Vinasse 15% (v v^{-1})	0,36 ± 0,01	1,92 ± 0,02	174,12 ± 3,03

The test with vinasse at a dilution of 10% (v v-1) was the one that guaranteed the highest biomass productivity according to the values shown in Table 2, reaching 186.01 ± 1.79 mg L-1 Day-1 while the control obtained a productivity of 150.67 ± 1.02 mg L-1 Day-1.

The cellular composition of the dry microalgal biomass was analyzed in order to check for the influence of the cultivation medium on the biochemical characteristics of Chlorella sorokiniana, using the biomass from the treatment with a 10% (v v-1) dilution of vinasse (Figure 5a) and the control (Figure 5b).

Figure 5

Biochemical composition of Chlorella sorokiniana cultivated with vinasse (10% v v-1) (A) or N:P:K (20:5:20) (B).

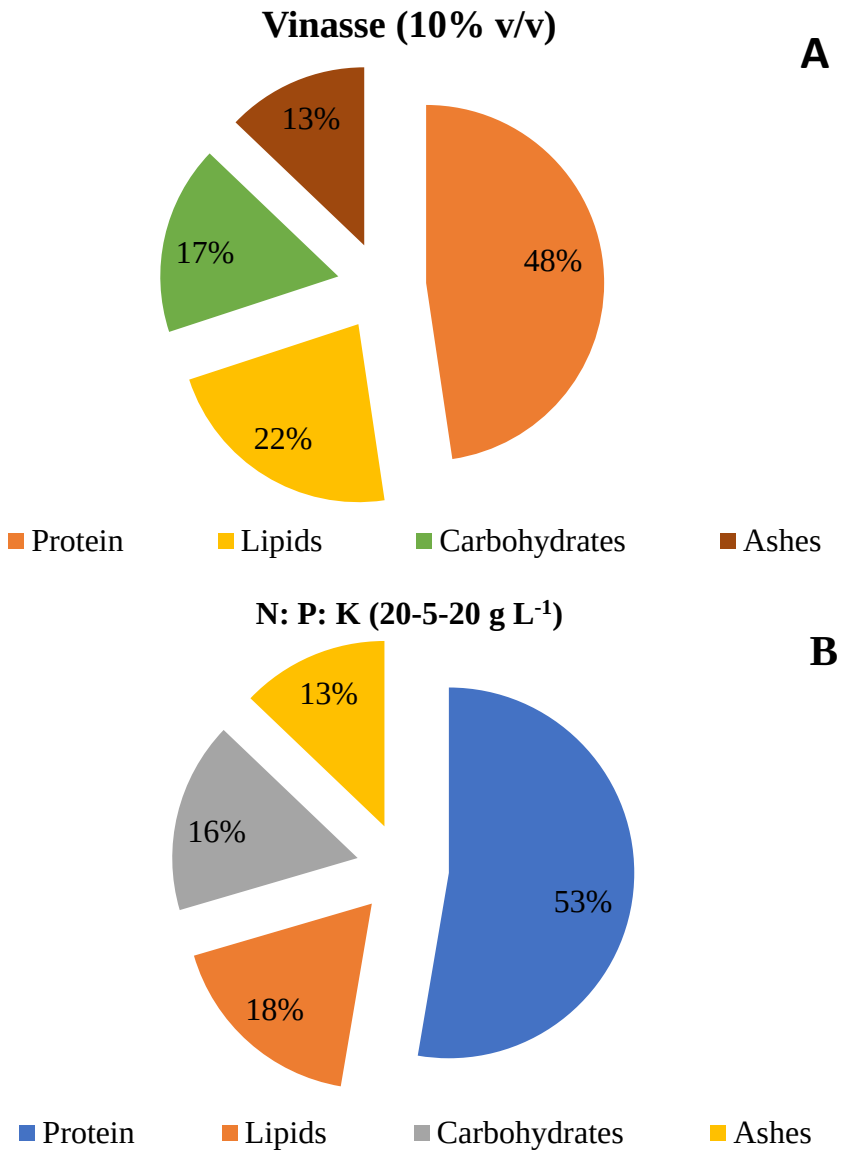


Figure 5 shows that a large proportion of the biomolecules present in the cell of Chorella sorokiniana are proteins, while the second highest concentration (22% in vinasse 10% (v v-1) and 18% in N:P:K (20:5:20)) is represented by lipids, indicating its potential for use in biodiesel production. In addition, it should be noted that the treatment with vinasse resulted in a higher lipid fraction than the control, which is possibly explained by the biochemical stress caused by vinasse, since N:P:K has optimal concentrations for the metabolism of microalgae while vinasse, although rich in nutrients, may not offer ideal concentrations for the nutritional requirements of Chlorella sorokiniana.

3.1 NUTRIENT REMOVAL DURING CULTIVATION

The cell growth of microalgae is influenced by a series of parameters, including light intensity and the concentration of nutrients in the cultivation medium. Considering the levels of ammonia, nitrite, nitrate and phosphate, it can be said that Chlorella sorokiniana achieved good removal results during the cultivation period, reaching removal values of more than 70% for all the nutrients evaluated in cultivation with 10% (v v-1) vinasse, as shown in the graph in Table 3.

Table 3

Initial and final nutrient concentration and removal rate.

Nutrient	N:P:K (20:5:20)			Vinasse 10% (v v^{-1})		
	Beginning (mg L^{-1})	End (mg L^{-1})	Removal (%)	Beginning (mg L^{-1})	End (mg L^{-1})	Removal (%)
Ammonia	589,42 ± 2,31	47,15 ± 0,89	92,00	67,24 ± 0,76	2,02 ± 0,24	97,00
Nitrite	345,67 ± 1,09	62,22 ± 0,74	82,00	1,43 ± 0,18	0,21 ± 0,03	85,00
Nitrate	643,18 ± 3,16	141,50 ± 1,01	78,00	44,16 ± 0,49	8,39 ± 0,31	81,00
Phosphate	167,48 ± 1,42	75,37 ± 0,93	55,00	2,19 ± 0,14	0,48 ± 0,08	78,00

The consumption of nutrients in microalgae cultures can be monitored by determining their concentration in the culture medium (Liyanaarachchi et al., 2021). As can be seen in Table 3, Chlorella sorokiniana achieved promising results in the removal of three of the four nutrients evaluated. In the treatment with vinasse, there was a considerable depletion of the concentration of ammonia in the medium, which had an initial concentration of 67.24 mg L-1 and reached 2.02 mg L-1 at the end of the experiment, achieving a removal equivalent to 97%.

Regarding ammonia depletion, Dinnebier et al. (2021) analyzed the remediation of ammonia nitrogen in the cultivation of Chlorella sorokiniana in pig digestate, which varied from 988 mg L-1 at the start of the experiment to 187 mg L-1 at the end, a removal equivalent to 81%, lower than that achieved in this work.

In the phytoremediation of vinasse using microalgae, ammoniacal nitrogen is shown to be the preferred compound to be incorporated by the cell, because its assimilation requires less energy, since it does not require a redox reaction and is assimilated naturally by the microalgae (Jiang et al., 2011). This justifies the better removal results obtained by ammonia compared to nitrite and nitrate.

Considering the treatment with vinasse, the nitrite concentration varied from 1.43 to 0.21 mg L-1 at the end of 30 days of cultivation, a removal percentage equivalent to 85%. 28 They analyzed nitrite removal by the microalga Chlorella vulgaris cultivated in different dilutions of a residual hydroponic solution at an average temperature of 25ºC and constant lighting. Cultivation ensured a depletion in nitrite concentration equivalent to 84.5%, ranging from 0.27 mg L-1 at the start to 0.04 mg L-1 at the end. These results are similar in percentage removal to those of this study and indicate the potential of applying microalgae to remediate this compound in various effluents.

Nitrate is the compound with the lowest assimilation affinity for algae when considering the different forms of inorganic nitrogen, a fact confirmed by Drexler et al. (2014) where the authors evaluated the nitrate and ammonia nitrogen removal capacity of Chlorella sorokiniana grown in Wastewater Treatment Plant (WWTP) effluent for 12 days. Removal rates equivalent to 10% were obtained for nitrate (3.50 mg L-1 at the start and 3.15 mg L-1 at the end of cultivation); on the other hand, ammonia nitrogen removal was 98% (24.00 mg L-1 at the start and 0.48 mg L-1 at the end of cultivation), a behavior similar to that presented in this study, again confirming the metabolic preference for ammonia in microalgae. It should be noted that the absolute values and the percentage of removal differed from those in this study, a fact explained by the shorter cultivation time, which in this study was equivalent to 30 days.

With regard to phosphate concentration, for cultivation with 10% vinasse (v v-1) it varied from 2.19 mg L-1 to 0.48 mg L-1, showing a removal rate of 78%. These results are lower than those obtained by29 where the phosphate content ranged from 32.00 mg L-1 at the start of cultivation to 1.28 mg L-1 at the end of 12 days of cultivation, removing around 96% of phosphate from Chlorella sorokiniana cultivated in WWTP effluent.

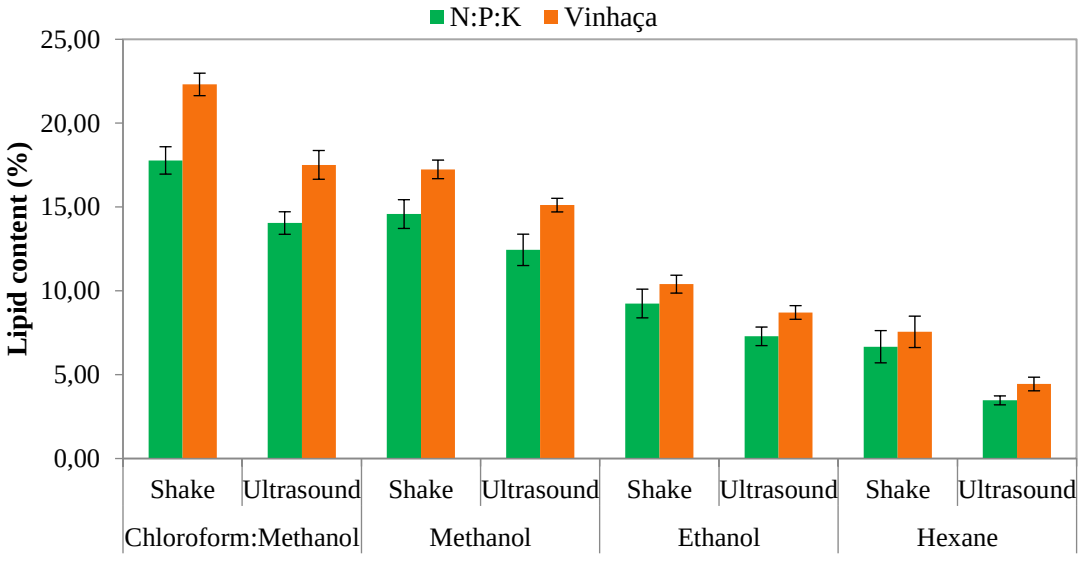
The percentage of phosphate removal was the lowest in relation to the other nutrients analyzed, which corroborates the study of 30 highlighting that the removal of nitrogenous compounds by microalgae is more efficient than phosphorus compounds.

3.2 INFLUENCE OF THE SOLVENT ON LIPID EXTRACTION

According to Richmond (2004); Nichols (1965) and Becker (2004) microalgae of the genus Chlorella reach a lipid content in the range of 20 to 30% of their dry weight when cultivated under controlled conditions, i.e. temperature, light intensity and nutrient concentration in the medium that favor their development. Considering Chlorella sorokiniana, lipid extraction was evaluated using different methods, combining cell disruption and chemical solvents, in order to determine which would guarantee the greatest extraction potential. The yields obtained showed differences in each model analyzed and are shown in the graph in Figure 6. It should be noted that for analysis of the lipid content and biodiesel production, the dry biomass from cultivation with 10% v v-1 vinasse was used, since it was the biomass that guaranteed the best results in cultivation.

Figure 6

Yields obtained in the different lipid extraction models evaluated.



Analyzing the results shown in the graph, it can be concluded that vinasse proved to be an excellent medium for cultivating microalgae, especially the Chlorella sorokiniana species, since in addition to ensuring good cell growth results, it also provided an increase in the

intracellular lipid fraction, as shown in Figure 6. This can be explained by the biochemical stress that the medium causes on cell development, since it is rich in nutrients, but has a low concentration of nitrogen, a limiting nutrient for the development of proteins in the cell (Adarme-Vega et al., 2012)

As shown in Figure 6, the best yield was obtained from the Chloroform:Methanol mixture with magnetic stirring, reaching a content of 17.78 ± 0.81 and 22.31 ± 0.67% for the test with N:P:K (20:5:20) and 10% (v v-1) vinasse, respectively. These values confirm the selectivity of the solvent in the extraction of intracellular lipids. This mixture is used in the method of D'oca et al. (2011) and is capable of extracting all classes of lipids present in the cell, since it combines a polar solvent with an apolar one, increasing its selectivity and, consequently, increasing yields. However, this conventional method is limited to its unfeasibility in industrial application, due to the high solvent costs and high toxicity (D'Oca et al., 2011; Adarme-Vega et al., 2012)

Checking all the contents shown in Figure 6, it can be said that magnetic stirring proved to be more effective than ultrasound, guaranteeing more promising results in most of the tests. This can be explained by the greater homogenization of the medium and the cell shock caused, ensuring a higher solvent-solute ratio, thus resulting in greater extraction Adarme-Vega et al., 2012).

3.3 FATTY ACID PROFILE AND COMPOSITION

The biodiesel from the microalga Chlorella sorokiniana grown in vinasse was subjected to chromatographic analysis in order to determine the main fatty acids (FAs) present in it. The mass quantification was normalized as a function of the total fatty acids identified, which in this study were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3), as shown in Table 4. The quantification of fatty acids was compared to other conventionally used matrices, in order to provide a standard of comparison for the biodiesel produced.

Table 4

Identification and quantification of biodiesel fatty acids.

Fatty acid	*Chlorella sorokiniana (%)*	Soybean (%)	Palm (%)
C14:0	1,16	0,00	1,10
C16:0	44,18	11,40	42,70
C16:1	4,15	0,00	0,00
C17:0	0,00	0,00	0,20
C18:0	3,19	3,60	4,50

C18:1	23,42	25,20	39,40
C18:2	16,54	53,60	10,60
C18:3	5,69	6,20	0,10
C20:0	0,00	0,00	0,39
C20:1	0,00	0,00	0,20
C22:0	0,00	0,00	0,60
C24:0	1,67	0,00	0,10
C24:1	0,00	0,00	0,10
Ref	Autor	Ramos et al., (2009)	Ramos et al., (2009)

As can be seen in Table 4, the smallest portions identified in Chlorella sorokiniana biodiesel were C14:0 and C24:0, a characteristic in line with the literature D'oca et al. (2011) and Prabakaran; Ravindran (2011), which points out that the percentage of these FAs does not exceed 2% in freshwater microalgae. In a similar way to higher plants, the most abundant fatty acids in microalgae such as Chlorella are found in the C16 to C18 range, corroborating the results obtained and presented in Table 4.

The fatty acid profile and the fraction of fatty acids present is of fundamental importance for the quality of biodiesel. The most conventional fatty acids in biodiesel, according to Knothe (2005), are palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linolenic acid (C18:3), validating the results obtained in this study, since the largest portions correspond to the aforementioned fatty acids.

There are a number of properties that have a direct impact on the quality of biodiesel, some of the main ones being the amount of each FAME, the length of the FFA chain and the number of double bonds (Chisti, 2007 and Knothe, 2005). Considering the saturated FAs most present in the biodiesel studied (C14:0, C16:0 and C18:0), it should be noted that they are more prone to solidification at low temperatures, influencing the properties of Cold Filter Plugging Point (CFFP) and Cloud Point (CP).

3.4 PROPERTIES OF THE BIODIESEL PRODUCED

The biodiesel produced was analyzed and its properties were estimated empirically. The parameters determined were the degree of unsaturation, cetane number, saponification index, iodine index, saturated long chain factor, cold filter plugging point, cloud point, calorific value, viscosity and specific mass. In order to confirm the quality of the biodiesel produced, it was compared with other conventional matrices, such as soybean oil and other microalgae, as shown in Table 5.

Table 5

Properties of biodiesel from microalgae and soybean oil.

Properties	Standart	*Chlorella sorokiniana (Este trabalho)*	*Chlorella sorokiniana*	*Chlorella vulgaris*	Soybean oil
SFA (%)	-	50,20	48,03	52,15	15,00
MUFA (%)	-	27,57	24,64	37,51	24,70
PUFA (%)	-	22,23	27,00	10,33	60,30
DU	-	72,03	78,64	58,17	-
SI (mg L^{-1})	-	206,14	208,06	61,83	51,70
II (g I_2/100g)	-	80,87	81,95	199,37	-
NC	-	46,32	54,09	52,63	-
SLCF	-	9,35	8,29	1,57	-
CFPP (ºC) *	8,00 – 14,00	12,91	9,57	-10,81	-
CN (ºC)	-	18,25	15,88	-	0,00
PC (MJ Kg^{-1})	-	38,97	39,18	-	39,79
V (mm^2 s^{-1})	3,00 – 6,00	4,10	3,65	-	4,10
D (g cm^{-3})	0,85 – 0,90	0,88	0,87	-	0,81
Ref	-	Autor	Menegazzo et al., (2020)	Nascimento et al., (2020)	Ramirez et al., (2012)

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, DU: degree of unsaturation, SI: saponification index, II: iodine index, CN: cetane number, SLCF: saturated long chain factor, CFPP*: cold filter plugging point in MS, PN: cloud point, PC: calorific value, V: kinematic viscosity, D: specific mass.

Biodiesel from Chlorella sorokiniana complies with ANP regulation 45/2014, since all the parameters calculated were in accordance with the limits established by the regulation. This underscores the potential of using the fatty acid profile and composition to apply empirical equations, making it possible to estimate the specificities of biodiesel from this species of microalgae, corroborating the study of (Menegazzo et al., 2017).

Regarding the fatty acid profile, Chisti (2007) points out that the concentration, length of the carbon chain and number of unsaturations directly influence the parameters of biodiesel. The degree of unsaturation is indicated by the Iodine Index (II), which increases proportionally to the increase in the amount of unsaturated fatty acids (Chuah et al., 2016; Menegazzo et al., 2015). In this study, the II was moderate, in contrast to soy biodiesel Ramírez-Verduzco et al. (2012) and Ramos et al. (2009), which is explained by the higher proportion of saturated fatty acids.

The length of the chain, the degree of unsaturation, the number of double bonds and the molecular weight of the AGs all influence the behavior of the Cetane Number (CN). The CN refers to the fuel's burning potential after injection; the higher the CN, the better the ignition capacity and the lower the generation of atmospheric pollutants (Wang et al., 2012). The NC

presented by Chlorella sorokiniana is within the range presented by microalgae of the same genus and complies with current regulations.

According to Knothe (2005); Chuah et al. (2016) and Ramírez-Verduzco et al. (2012), calorific value (CP) is conventionally used to determine the energy content and efficiency of fuel. This parameter reflects the amount of heat released in the combustion of 1 g of fuel to generate carbon dioxide and water. The results of a study carried out by the National Institute of Biofuels report that the CP of microalgae is in the range of 36 to 39 MJ Kg^{-1}, which corroborates the value determined for the biodiesel in this study, confirming its quality and potential for use.

According to ANP 45/2014, viscosity and specific mass must fall within the range of 3.0 to 6.0 mm_2 s^{-1} and 850 to 900 Kg $m-^{3}$, respectively. In the biodiesel in this study, the viscosity was equivalent to 4.10 mm^2 s-1 and the specific mass was 0.88 g cm^{-3}, which is in line with the current standard, reflecting its capacity for use and efficiency of yield, since these parameters have a direct impact on fuel consumption, since the lower their values, the less fuel will be injected, providing the same yield with smaller volumes.

The standard established for the Cloud Point (NP) shows the recommended temperature so that the fuel does not start a solidification process (Talebi et al., 2013). The Cold Filter Clogging Point (CFCP), on the other hand, reflects the temperature at which crystallization or solidification occurs which is sufficient to cause the fuel filter to clog (Islam et al., 2013). These two parameters, according to the manufacturer, determine the fuel efficiency of the fuel. These two parameters determine the temperature limits capable of guaranteeing the liquid state of biodiesel, and vary depending on the country, region and climatic season (Chuah et al., 2016). The biodiesel produced in this study has potential for use as B100 in the state of Mato Grosso do Sul, since its required PEFF range is between 8 and 14ºC.

With regard to the standards regulated by ANP 45/2014, biodiesel from Chlorella sorokiniana grown with diluted vinasse is of a quality that complies with what is established for use as B100 in the state of Mato Grosso do Sul, making it a promising alternative to fossil fuels, especially diesel oil, as well as to other biodiesels produced from conventional crops, such as soybean oil.

4 CONCLUSIONS

Given the circumstances expressed here, it can be said that the microalgae species cultivated with diluted vinasse under the treatment conditions presented proved to be an interesting option for biodiesel production, as it ensured good adaptation to the environment, remediation of the industrial effluent and use of its nutrients in its cellular development, a considerable increase in its lipid content, as well as a biofuel that complies with national regulations, attesting to its potential for this purpose.

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APPENDICES

Table 6

Specification of the formulas.

Equation	Specifications	Formula
1	E= Nutrient removal rate (%) X_2 – Nutrient concentration at the start of cultivation; X_1 - Nutrient concentration at the end of cultivation.	$E\ (\%) = \left(1 - \frac{X_2}{X_1}\right) * 100$
2	CN= cetane number; IS= Saponification Index; II= Iodine Index.	$CN = 46{,}3 + \left({}^{5{,}458}/_{IS}\right) - \left({}^{0{,}225}/_{II}\right)$
3	SI= Saponification Index Mi=represents the value of the molecular mass of the fatty ester; Ni= percentage of the particular fatty ester in the oil sample.	$SI = \sum {}^{(560\ x\ Ni)}/_{Mi}$
4	II=Iodine Index; Mi=represents the value of the molecular mass of the fatty ester; Ni= percentage of the particular fatty ester in the oil sample.	$II = \sum {}^{(254\ x\ D\ Ni)}/_{Mi}$
5	DU= Degree of Unsaturation AMFA= amounts of monounsaturated fatty acids APFA= amounts of polyunsaturated fatty acids	$DU = AMFA + (2\ x\ APFA)$
6	SLCF= Saturated Long Chain Factor	$SLCF = (0{,}1\ x\ C16) + (0{,}5\ x\ C18) + (1{,}0\ x\ C20) + (1{,}5\ x\ C22) + (2{,}0\ x\ C24)$
7	CFPP= Cold Filter Plugging Point	$CFPP = (3{,}1417\ x\ LCSF) - 16{,}477$
8	CP= Cloud Point	$CP = (0{,}526\ x\ C16) - 4{,}992$
9	v = Kinematic viscosity; Mwi = the molecular weight of the fatty acid; Ni = percentage of fatty acid; Di = number of double bonds present in the given fatty acid.	$\ln(v) = \sum Ni(-12{,}503 + (2{,}496\ x \ln Mwi) - 0{,}178\ x\ Di)$
10	ρ = specific mass; Mwi = the molecular weight of the fatty acid; Ni = percentage of fatty acid; Di= amount of double bonds present in the given fatty acid.	$\rho = \sum Ni(0{,}8463 + (4{,}9/Mwi) + 0{,}0118\ x\ Di)$
11	CV= Calorific Value; Mwi = the molecular weight of the fatty acid; Ni = percentage of fatty acid;	$CV = \sum Ni(46{,}19 - (1794/Mwi) - 0{,}21\ x\ Di)$