EXPLORING THE HETEROTROPHIC CULTIVATION ON SUGARCANE VINASSES BY *Chlorella vulgaris*

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ABSTRACT

The recovery of resources from agro-industrial waste from the sugar and alcohol industry, such as vinasse, is an important strategy to promote sustainability and mitigate negative environmental impacts. In this scenario, microalgae represent a potential alternative for the biological treatment of sugarcane vinasse. In the current study, vinasse was used as a culture medium for the microalga *Chlorella vulgaris* under heterotrophic growth conditions. The experiments were conducted at different vinasse concentrations (1.72% to 58%) and *C. vulgaris* inoculum concentrations (0.1 to 0.8 g/L). The maximum biomass productivity achieved was 215.71 mg/L.d, with a phosphorus removal efficiency of 67.7%. Additionally, the resulting algal biomass (microalga biomass + vinasse) was tested for it is germination potential and toxicity in lentil seeds. Treatments with the microalga-vinasse mixture showed a 37% increase in seed germination (radicle and hypocotyl growth) compared to control (water). These results suggest that *C. vulgaris* can be effectively used to reduce contaminant load of vinasse and produce microalgal biomass, recovering a value-added product and offering a sustainable solution for waste treatment.

Keywords: Agro-industrial waste. Lentil seed. Microalgae. Phosphorus removal. Wastewater.

1 INTRODUCTION

Vinasse is one of the main wastewaters generated during the processing of sugarcane for food and energy, with an estimated generation raging from 10 to 15 liters per liter of distilled ethanol[1]. This wastewater is characterized by a high chemical oxygen demand (COD) of approximately 30,000 mg/L, as well as elevated levels of biological oxygen demand (BOD) (8,300 mg/L), phosphorus (105 mg/L), turbidity (110 NTU) and an acidic pH (~4.5)[1,2]. Improper disposal of vinasse can lead to significant environmental impact. To mitigate this, fertigation of crops is a common practice for its reuse. However, continuous use of vinasse on the soil can induce adverse effects, such as salinization, which affects the quality of agricultural crops.

Microalgae, with their versatility, are emerging as a promising solution for the treatment of industrial wastewater like vinasse. Microalgae-based processes have the potential to reduce the contaminant loads while producing valuable biomass. In addition, these microalgae can adapt to various environments and cultivation conditions, with (autotrophic or mixotrophic) or without light (heterotrophic)[1,3]. Given the high turbidity of vinasse, heterotrophic cultivation of microalgae, which does not require light, is a potential approach for treating this wastewater. This method allows the use of dark, turbid media and can facilitate control process parameters[1].

In this context, vinasse can be considered a sustainable and economical alternative for growing microalgae, promoting a circular bioeconomy by combining nutrient removal from wastewater with microalgal cell growth. This process generates value-added products such as biomass rich in proteins and lipids, pigments and fertilizer[4].

Given this context, the aim of this study was to evaluate the heterotrophic cultivation of the microalga *Chlorella vulgaris* using vinasse from the sugar-alcohol industry as a growth medium. Experiments were conducted using a Rotational Central Composite Design (RCCD) 2^2, evaluating vinasse content (1.72 to 58% v/v) and the microalgae inoculum concentration (0.1 to 0.8 g/L). After cultivation, germination tests on lentil seeds were performed to preliminarily assess the potential of the biomass as a biofertilizer and/or its possible toxic effect on the seeds.

2 MATERIAL & METHODS

The vinasse was sourced from a sugar and ethanol industry located in São Paulo, Brazil. Its pH was adjusted to 6.5 by adding 4N NaOH. Following the adjustment, the vinasse was centrifuged at 2700 rpm for 10 min to remove suspended solids. The supernatant was then autoclaved at 120°C for 20 min[5].

The microalga *C. vulgaris* was cultured in BG-11 medium and maintained at 26°C with a 12:12 light-dark photoperiod (50 µmol m^{-2} s^{-2}) until reaching the exponential growth phase[6].

For heterotrophic growth experiments, a CCDR 2^2 was employed, evaluating vinasse concentrations ranging from 1.72% to 58% (v/v) and *C. vulgaris* inoculum concentrations from 0.1 to 0.8 g/L[7]. The dependent variables assessed were productivity (mg/L.d) and phosphorus removal efficiency (%). The experimental setup was conducted in 250 mL flasks containing 100 mL of diluted vinasse in saline medium at the appropriate proportions. The flasks were covered with aluminum foil to prevent light exposure and were maintained under agitation at 100 rpm for 14 d. Microalgal productivity was quantified based on the dry weight (105°C) of the microalgae. Phosphorus concentration was determined using a colorimetric method (660 nm) with ammonium molybdate and ascorbic acid solution[8]. The samples were quantified using a standard curve prepared with KH_2PO_4.

The sample with the highest microalgal biomass productivity was further evaluated for germination potential and toxicity in lentil seeds (*Lens culinaris*)[9]. In brief, 7 treatments were conducted in which 3 mL of either microalgae-vinasse mixture or raw vinasse were diluted in water (1:25) and placed in Petri dishes containing 20 seeds arranged on filter paper. Control samples were conducted with distilled water. The plates were incubated at 20°C for 96 hours, with the sample volume replenished after 48 hours to prevent drying. Germination percentage was determined, and both radicle and hypocotyl lengths were measured. The tests were conducted in triplicate and statistically analyzed using ANOVA followed by Tukey's test ($p < 0.05$).

3 RESULTS & DISCUSSION

The microalgae *C. vulgaris* was used in this study (Figure 1) due to its metabolic versatility, robustness, tolerance, and ease of cultivation and strain management[3]. With extensive applications in environmental biotechnology, *Chlorella* shows great potential for large-scale processes. It can tolerate adverse environments and efficiently remove nutrients from wastewater, including domestic[10], livestock[11], and industrial[12].

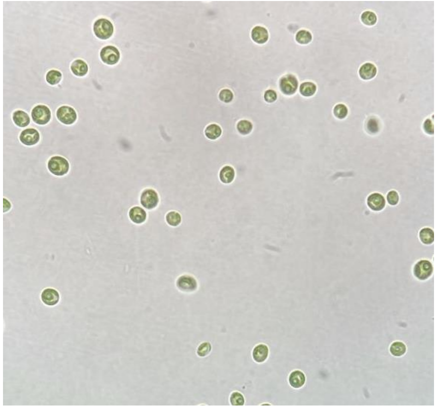

Figure 1 Microscopy image (100x) of *C. vulgaris*.

The growth of the microalgae *C. vulgaris* under heterotrophic conditions was evaluated with different concentrations of inoculum and vinasse. Table 1 presents the RCCD results evaluated in terms of biomass productivity (mg/L.d).

Table 1 Matrix of the experimental de design (code and real values) and the response in terms of microalgae productivity in 14 days

Experimental assay	Independents variable		Dependent variable	Phosphorus removal (%)
	Vinasse (%)	Inoculum (g/L)	Productivity microalgae (mg/L.d)	
1	-1 (10)	-1 (0.2)	49.29	35.36
2	+1 (50)	-1 (0.2)	83.57	52.58
3	- 1 (10)	+1 (0.7)	66.43	10.91
4	+1 (50)	+1 (0.7)	124.29	20.43
5	+ 1.41 (58)	0 (0.5)	215.71	67.67
6	-1.41 (1.72)	0 (0.5)	33.57	11.30
7	0 (30)	+1.41 (0.8)	144.75	53.85
8	0 (30)	-1.41 (0.1)	58.11	49.06
9	0 (30)	0 (0.5)	92.14	55.72
10	0 (30)	0 (0.5)	87.14	58.40
11	0 (30)	0 (0.5)	90.71	51.27

The presented data demonstrate that the microalga *C. vulgaris* has the potential to grow in vinasse, with the best condition being 58% vinasse and 0.5 g/L inoculum (215.71 mg/L.d). The interaction observed in our study indicates that vinasse concentration positively influenced the biomass productivity ($p_{value} < 0.05$) within the evaluated ranges. Comparable results were obtained by Candido and Lombardi[13], who observed higher microalga growth at a 60% vinasse concentration.

Soto et al.[5] evaluated the removal of nutrients from vinasse using *C. vulgaris* and observed a decrease of 21.45% and 49.13% in COD and BOD after 120 hours of cultivation, respectively. Additionally, they observed reductions of 61% in total organic carbon, 85% in total phosphorus, and 18% in sulfates. These findings support the results obtained in this study, where, under heterotrophic conditions, the toxic potential of wastewater can be reduced. In our study, the experiment with the highest productivity also showed the highest phosphorus removal (67.7%).

The growth of *C. vulgaris* in all experiments led to the usual increase in pH, starting at 6.5 and reaching 8.2 to 9.1 by the end of the cultivation periods. This increase can be attributed to several factors: the absorption of nutrients from the vinasse, the production of extracellular compounds, and the continuous agitation of the culture. The agitation enhances the interaction with air, facilitating the removal of carbon dioxide produced during cellular respiration[14].

From a circular economy perspective, the biomass productivity results from assay 5 (Table 1) suggest the potential of utilizing vinasse to produce *Chlorella*, which can be applied in low-cost agricultural practices, thereby reducing production costs associated with the cultivation medium. Considering this scenario, the next step was to evaluate the characteristics of the medium produced (vinasse + microalga) on lentil seeds.

The samples were diluted 1:25 (raw vinasse and vinasse + microalga) and lentil seeds were used in a toxicity assay. The results showed no statistical difference ($p_{value} > 0.05$) in germination between the control sample (water) and the vinasse samples. However, slightly higher radicle lengths were measured with exposure to vinasse + microalga (7.76 ± 1.27) compared to crude vinasse (5.49 ± 1.09) and water (5.68 ± 1.13), with a statistically significant difference ($p_{value} < 0.05$) between the sample with microalga and the others (control and raw vinasse).

Based on preliminary results, residual vinasse containing microalgal shows promise for agricultural crops, harnessing the growth benefits provided by vinasse and microalgal characteristics. This utilization of industrial wastewater as a microalgae cultivation medium, subsequently repurposed for biofertilizer production, aligns with green economy principles and reduces the water footprint of microalgal cultivation by decreasing the need for potable water.

4 CONCLUSION

This study demonstrates that the microalga species *C. vulgaris* can efficiently grow in vinasse under heterotrophic conditions (complete absence of light). The results showed a productivity of 215.71 mg/L.d with an initial inoculum concentration of 0.5 g/L and a vinasse content of 58% (v/v). Additionally, preliminary germination and toxicity tests on lentil seeds showed no significant difference in seed germination between the sample (vinasse + microalga) and the control (water), while radicle growth increased by 37%. These findings provide evidence that the use of algal biomass and vinasse has potential for remediation and fertilization in agriculture.

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