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ENVIRONMENTAL MICROBIOLOGY - RESEARCH PAPER

Bioprospecting and selection of tolerant strains and productive analyses of microalgae grown in vinasse

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Abstract

In order to contribute to the biotechnology of microalgae cultivated in vinasse, we carried out the bioprospection of tolerant species and synthesized biomolecules of the total biomass (microalgae and bacteria), recovered from cultures. To use vinasse as a culture medium for the microalgae, waste was centrifuged and used in concentrations from 5 to 50%. Daily cell densities, growth rates, and EC50 values were obtained. After defining the best pair of vinasse concentration/microalgae strain, dry biomass, and composition (proteins and carbohydrates) were determined in 96 h cultures, considering the associated community (bacteria and yeast). The microalgae tested were *Chlamydomonas* sp., *Chlorella sorokiniana*, *Chlorella vulgaris*, *Desmodesmus spinosus*, *Haematococcus pluvialis*, *Monoraphidium* sp., *Scenedesmus quadricauda*, and *Tetraselmis gracilis*. The results showed that although the microalgal growth rates in vinasse were similar to controls in BG11, the cells in vinasse had higher biovolumes, dry biomass, and total proteins. The species *H. pluvialis*, *S. quadricauda*, and *T. gracilis* showed the best productivity parameters in vinasse, despite lower growth rates than the other species. Using low concentrations of centrifuged vinasse as a culture medium, only 22% of biological contaminants were present, thus most of the processed biomass was mainly composed of microalgae. Thus, *Chlamydomonas* sp., *D. spinosus*, *S. quadricauda*, and *H. pluvialis* microalgae have attributes such as resistance and biomolecules that make them candidates for further optimization in production systems, combining the environmental benefits of using waste with the production of biomolecules and/or biomass of commercial interest.

Keywords Phytoplankton · Cultures in wastes · Vinasse · Biomass production · Biovolumes · Biomolecules

Introduction

Vinasse is waste from the sugar-alcohol industry with high amounts of organic and mineral nutrients, high conductivity, biochemical, and chemical oxygen demands and dissolved organic carbon [1]. It is estimated that approximately 384 billion liters of sugarcane ethanol vinasse were produced by biorefineries in Brazil between 2016 and 2017 [2]. These characteristics make vinasse highly polluting waste, hence the prohibition to discard it in aquatic environments. Vinasse is currently used in soil fertirigation, but this practice can salinize the soil and contaminate aquatic ecosystems by flow and percolation [1]. Thus, alternative uses for vinasse are a contribution to sustainable green energy production, such as microalgal cultivation. In addition to generating commercially valuable biomass [3], the cultivation of microalgae in vinasse enables bioremediation of the waste [4], while sequestering atmospheric carbon, helping to mitigate the greenhouse effect [5].

Most of the research available in the literature on microalgal growth in vinasse is carried out with strains of *Chlorella vulgaris*, *Scenedesmus* sp., and *Spirulina* sp. In common, the organisms that grow in vinasse need to be robust enough to support the adverse conditions of the waste, such as high osmolarity, a great variety of toxic compounds and its low pH [1, 6, 7]. *C. vulgaris* is the most used species possibly because of its capacity to take advantage of organic components in vinasse through mixotrophy or heterotrophy [7–9].

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Many studies show that treating and/or diluting the vinasse before using it as part of microalgae culture medium can greatly enhance the ability of cells to grow in it [4, 10–16]. Some studies used the supernatant of centrifuged corn vinasse for the cultivation of microalgae [6, 17, 18]. However, from the published literature on microalgal growth in vinasse, we became aware that most of it attributes only to microalgae the totality of biomass produced. However, the biomass in organic waste as vinasse is not limited to that inoculated, but contains biological contaminants as bacteria and fungi [4], which can account for a great percent of the total biomass computed.

In general, studies in the literature that show the cultivation of microalgae in vinasse use only one or few species, different waste pretreatments, and measures to represent microalgal production, disregarding the biological contamination of this total. There is much interest in the growth of microalgae in waste because it can remediate them and, at the same time, reduce microalgae production costs. However, according to the literature, there are still several gaps that make it difficult to optimize the biotechnological use of vinasse in microalgal cultivation. Among them, the knowledge of strains that present high resistance to high concentrations of vinasse. In the study by Falconí et al. [15], 10 green microalgae (*Chlorophyta*) were screened in different culture media based on sugarcane ethanol vinasse. The growth performance results clearly showed that only *C. sorokiniana* BR001 and *S. obliquus* BR003 were able to present growth rates above 0.3 day^{-1}. Therefore, more research is needed aiming at the bioprospection of microalgal strains tolerant to relevant concentrations of vines and the synthesis of biomolecules.

In this context, the objective of this research was to prospect different species of freshwater and marine microalgae regarding their growth capacity and tolerance in optimal concentrations of vinasse and its biomolecules. This considers the biological load present in vinasse, which grows in consortium with microalgae.

Material and methods

Vinasse, microalgae strains, and physicochemical characterization of the effluent

The vinasse used in this study was collected from the São João plant (Araras, Brazil) and the unique treatment prior to inoculating the microalgae was centrifugation. It was centrifuged in a refrigerated centrifuge (Sorvall-Thermo, Legend XTR model, USA) at 4000 rpm (2510 g) and 25 °C for 15 min. Vinasse centrifugation was adopted because it is more economically and environmentally viable compared to other vinasse pretreatments proposed in the literature and maintains the chemical characteristics of raw vinasse [12]. This vinasse pretreatment is a physical process that reduces particulate materials, including yeasts that cause shading effects and compete with the microalgae for nutrients [12]. The centrifuged vinasse was not autoclaved or sterilized. The vinasse treated by centrifugation was physicochemically characterized at the ASL Environmental Analysis-Laboratory St. Luke (Rio Claro, SP, Brazil). The methods used by the laboratory followed the methodology proposed by the Brazilian Association of Technical Standards (ABNT 22ª Ed, 2012). The following parameters were analyzed: Total Suspended Solids (2540 D); Calculated hardness (2340 B); Electrolytic Conductivity (2510 B); Chemical oxygen demand-COD (5220 D); Biochemical oxygen demand-BOD (5210 B); Total Kjeldahl Nitrogen (4500-Norg B); Anions (POPDAM054); and Total Metals (3030 E). The physicochemical parameters determined for the centrifuged vinasse are shown in Table 1. For the sake of comparison, we report the composition of the BG11 medium used in the experiments.

The microalgal species used and their origins are listed in Table 2. The choice of strains of different groups and origins was thought to be as a way to involve various metabolic and adaptive capabilities to face the harsh conditions imposed by vinasse.

Toxicity and culture conditions

Toxicity tests were carried out for a duration of 96 h to screen 10 microalgae strains and the best vinasse concentrations for the development of the tolerant species. The cells were cultured in centrifuged vinasse at concentrations of 5, 10, 15, 20, 30, 40, and 50%. Previous tests had shown that there was no algae growth at concentrations higher than 50% centrifuged vinasse, therefore limiting the tests to that

Table 1 Physicochemical composition of centrifuged vinasse and chemical composition of BG11 medium

Parameter (mg L^{-1})	Vinasse	BG11
Total suspended solids (TSS)	1220.0	–
Calculated hardness	1573.6	–
Electrolytic conductivity (μS cm^{-1})	11,620.0	–
Chemical oxygen demand (COD)	44,863.0	–
Biochemical oxygen demand (BOD)	17,011.0	–
Total Kjeldahl nitrogen (TKN)	340.5	1116.6
Sulfate (SO_4-)	1416.0	29.0
Phosphate (PO_4-)	< 2.0	5.6
Sodium (Na)	104.0	421.0
Calcium (Ca)	648.0	10.0
Potassium (K)	4380.0	7.0
Magnesium (Mg)	498.0	7.0

Table 2 Microalgae strains with information origin environments from which they were isolated and culture collection where they were maintained

Species	Origin	Culture collection
Chlamydomonas sp.	Acid environment (pH 36)	Phycology Laboratory, Federal University of Santa Catarina (UFSC), Brazil
Chlorella sorokiniana	Freshwater	Institute of Plant Biochemistry and Photosynthesis, University of Sevilha, Spain
Chlorella vulgaris	Sewage Treatment Station (STS)	Laboratory of Algae Biotechnology, Federal University of São Carlos (UFSCar), Brazil
Monoraphidium sp.	Vinasse	
Cyclotella sp.	Freshwater	Laboratory of Phycology, Federal University of São Carlos (UFSCar), Brazil
Desmodesmus spinosus	Freshwater	
Haematococcus pluvialis	Freshwater	Department of Botany, University of British Columbia, Canada
Scenedesmus quadricauda	Freshwater	
Nanocloropsis gaditana	Open ocean	Oceanographic Institute, São Paulo University (USP), Brazil
Tetraselmis gracilis	Estuary environment	

maximum concentration. For freshwater species, vinasse was diluted with autoclaved distilled water and, for the marine algae, distilled water containing 30 g L^{-1} of commercial sea salt (Blue Treasure, Norway) was used. As controls, freshwater microalgae were grown in BG11 medium [19] and the seawater species (*N. gaditana* and *T. gracilis*) in BG11 with 30 g L^{-1} of seawater salt (Blue Treasure, Norway). For the freshwater diatom *Cyclotella* sp., 1.5×10^{-4} M of $NaSiO_3$ was added to the BG11 medium and vinasse treatments, providing enough silica for the frustule synthesis [20]. The initial pH of all cultures was adjusted to 6.8–7.0, except for *Chlamydomonas* sp., isolated from an acid environment, for which the pH was initially adjusted to 4.8–5.0.

Cultures were performed in batch mode with three experimental replicates in 250 mL tissue culture flasks with a ventilated lid, each containing 150 mL of culture medium, and were maintained at 25 ± 2 °C. Light fluorescent tube lamps intensities were measured in the flasks with a quantum light sensor (Spectrum Technologies, UK), and adjusted to 130 µmol of photons m^{-2} s^{-1} for most algae, regardless of the vinasse concentration. *Cyclotella* sp. and *H. pluvialis* cultures were illuminated with 90 µmol of photons m^{-2} s^{-1}. Therefore, the culture flasks were distanced differently from the light source, so that the higher the vinasse concentration, the closer they were to the light. This brightness adjustment was necessary because vinasse has a brownish color. The initial cell densities for all microalgae were 5.0×10^4 cells mL^{-1}, except for *H. pluvialis*, whose cultures were inoculated with mature cysts at a concentration of 2.5×10^4 cysts mL^{-1}. The inoculum was obtained from exponentially growing cells in BG11 medium with no vinasse.

Viability and cell density

Daily measurements of viable algal cells were made on an automatic counter Muse Cell Count & Viability Assay (Merck Millipore, USA). Growth rates were obtained by linear regression of the exponential growth phase using the viable cell densities. The EC50 values, which represent the concentrations of vinasse that inhibited algal growth by 50%, were calculated considering the average growth rates for the three replicates in vinasse treatments in relation to the average growth rates of the respective controls, allowing an ecotoxicological analysis of the waste.

From these experiments, the species with the highest growth rate and the respective optimal vinasse concentrations were selected to conduct more detailed experiments on the microalgae productivities, biomass biochemical composition and biological contaminants. The same experimental conditions described above were used throughout.

The cultures (96 h) were monitored daily for cell densities by counting in the Fuchs-Rosenthal chamber under an optical microscope (Nikon, Eclipse E200, Japan) with a total magnification of × 400. The manual count at this stage allowed for the daily visualization of the cultures for cell morphology and biological contaminants, in addition to algae cell counts. Growth rates (day^{-1}) in the exponential phase were calculated. Absorbance at 570 nm and at 684 nm were obtained in a spectrophotometer (NANOCOLOR UV/VIS, Macherey–Nagel, USA). Absorbance at 570 nm refers to the amount of particulate material in the medium [21], including microalgae and contaminants such as fungi and bacteria, while absorbance at 684 nm refers to molecules with chlorophyll *a* [22], such as microalgae. Therefore, comparing both values, we inferred the contaminant contribution to the total algal biomass.

The centrifuged vinasse, even when diluted, shows coloring and turbidity, which may modify the measurement cited above. Therefore, to carry out absorbance measures, samples of 4 mL of each culture were collected and centrifuged at 4000 rpm (2510 × *g*) and 20 ºC for 15 min. The supernatants were discarded. The pellets were resuspended in 4 mL BG11 medium and submitted to the spectrophotometric analysis. This same process was performed with the controls in BG11

so that the changes caused due to the described process could be identified.

In 96 h, measurements of the dimensions of 30 microalgae cells per treatment were made and used to calculate the cell biovolume (μm^3 $cell^{-1}$) according to the formulas proposed by Hillebrand et al. [23]. The total biovolumes (cm^3 mL^{-1}) of the cultures were obtained by multiplying the average cell biovolume by the respective final cell densities.

Proteins and carbohydrates

Dry biomass, total protein, and total carbohydrate analyses were performed at 96 h cultures considering the consortium, e.g., all organisms present in the cultures, mostly bacteria, yeasts, and other fungi in addition to microalgae. For dry biomass (mg L^{-1}), 30 mL from each culture were filtered through pre-weighed glass fiber filters with 47 mm diameter and 0.6 µm porosity. They were dried at 40 ºC for 48 h and then weighed again. Total proteins (mg L^{-1}) were performed in 50 mL cultures, extracted according to Rausch [24], with sodium hydroxide (NaOH) at 80 ºC, and determined according to the protocol described in Bradford [25], using the Coomasie Brilliant Blue reagent and measuring the solution absorbance at 595 nm in a spectrophotometer (NANOCOLOR UV/VIS, Macherey–Nagel, USA). Total carbohydrates (mg L^{-1}) were determined in 30 mL samples and quantified by Albalasmeh et al. [26], using H_2SO_4 and measuring the supernatant absorbance at 315 nm in the spectrophotometer. From these quantifications, we obtained the protein/carbohydrate (P/C) ratios.

Statistical analysis

Statistical analyses were performed using the R 3.3.1 program, with the ANOVA and Tukey parametric tests ($p < 0.05$) to compare the values of each parameter for all microalgae tested. Using the Excel program (2016), Student's *t* tests were performed to compare microalgae cultures at optimal vinasse concentrations with the respective controls.

Results and discussion

Toxicity

Figure 1 shows the growth rates and viable cell density at 96 h of cultivation as a function of the vinasse concentrations and Fig. 2 shows EC50 values of centrifuged vinasse concentrations. From these results, the optimal concentrations of vinasse to the microalgal development were 20% for *C. vulgaris* and *D. spinosus*, 15% for *C. sorokiniana*, and 5% for the other species. *C. sorokiniana*, *C. vulgaris*, and *S. quadricauda* presented growth rates in the optimal vinasse concentrations higher than their controls. Ryan et al. [27] and Mohana et al. [28] related the toxicity and dark color of distillery waste such as vinasse to the presence of phenolic compounds called melanoidins. Our results show that 8 of the 10 species initially tested were able to overcome this difficulty.

The best growth performances in vinasse were obtained by *C. sorokiniana* and *C. vulgaris.* The first was grown in 15% vinasse and the second in 20% with growth rates of 2.0 and 1.5 day^{-1} respectively. The genus *Chlorella* is recognized for its robustness and adaptability to environments with different salinities, inorganic and organic nutrients, temperatures and pHs [7]. Their rapid growth in vinasse may have favored them in the competition with contaminants such as bacteria and yeast, which were not totally removed by the centrifugation. Candido and Lombardi [12] obtained similar results for *C. vulgaris* with a growth rate of 1.2 day^{-1} in 60% of vinasse adsorbed by smectite clay and active carbon, one of the best values obtained by this species found in the literature. Although *C. vulgaris* has already been identified as a promising species to grow in vinasse [16, 18, 29], our work points out that *C. sorokiniana* can also produce good results.

Growth rates at optimal vinasse concentrations for *Chlamydomonas* sp., *D. spinosus*, and Monoraphidium sp. were up to 50% lower than the controls and for *H. pluvialis* and *T. gracilis* were similar to controls. Despite the high reproductive capacities of *Chlamydomonas* sp., *D. spinosus*, and *Monoraphidium* sp. in the BG11 controls, their EC50 values did not exceed 20% of vinasse, indicating their high sensibility to the waste. However, considerable growths were obtained at optimum concentrations of vinasse. Similarly, Kadioglu and Algur [6] showed an increase in growth of *Chlamydomonas reinhardii* in TAP medium supplemented with 1–5% vinasse, but a growth reduction in higher vinasse concentrations. The authors achieved 5.0×10^5 cells mL^{-1} in a 96 h culture in TAP medium supplemented with 1% vinasse, while in the present research we reached 7.0×10^5 cells mL^{-1} in 5% vinasse diluted with water, a cheaper diluent. Considering *Desmodesmus*, Silva et al. [30] obtained a maximum growth rate of 0.045 h^{-1} (1.08 day^{-1}) for *D. subspicatus* in autoclaved vinasse grown under heterotrophic conditions, while we obtained 0.8 day^{-1} for another species, *D. spinosus,* under mixotrophic conditions. We found no literature addressing the cultivation of *Monoraphidium* sp. in vinasse. However, Yu et al. [31] obtained growth rates of 0.52 day^{-1} for *Monoraphidium* sp. in BG11 culture medium added with glucose and 0.15 day^{-1} under autotrophic conditions. Thus, despite our reduced growth for *Monoraphidium* sp. in vinasse (1.5 day^{-1} in BG11 medium and 0.8 day^{-1} in centrifuged vinasse), this result is significant when compared to the literature.

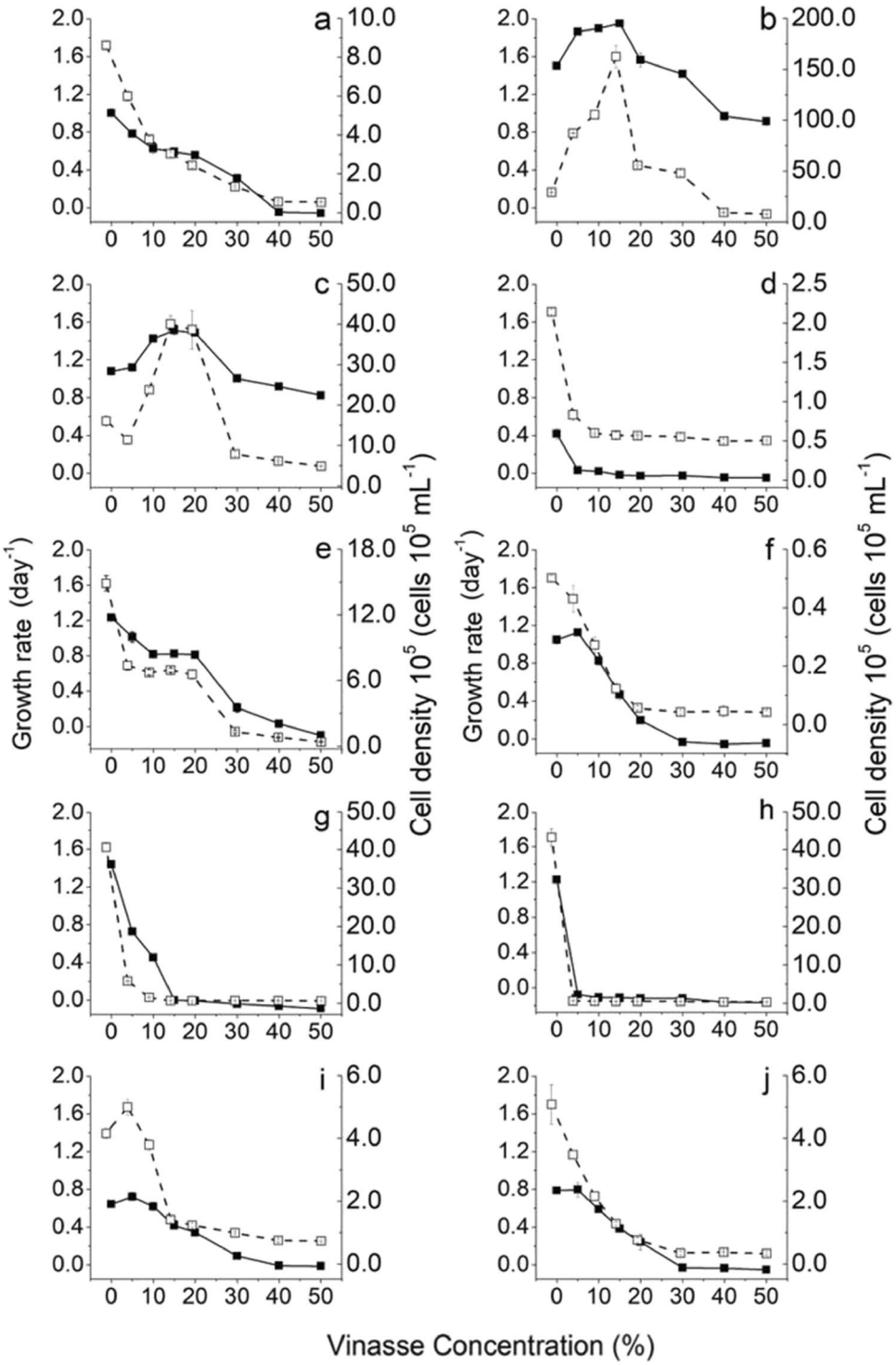

Fig. 1 Growth parameters: ( ) growth rates (day^{-1}) and () cell densities (cells 10^5 mL^{-1}) at 96 h for the vinasse concentrations (%) in cultures of **a** *Chlamydomonas* sp., **b** *C sorokiniana*, **c** *C vulgaris*, **d** *Cyclotella* sp., **e** *D spinosus*, **f** *H pluvialis*, **g** *Monoraphidium* sp., **h** *N gaditana*, **i** *S quadricauda*, and **j** *T gracilis*. The controls are represented in 0% vinasse

Even with Chlamydomonas sp. tolerance; *D. spinosus*, *H. pluvialis*, *S. quadricauda*, and *T. gracilis* did not exceed 20%. This result demonstrates the potential of these strains to be cultivated in vinasse using biochemical manipulation techniques. As demonstrated by authors such as Montalvo et al. [13] who diluted Vinasse in 30% water and obtained growth of 0.23 day^{-1} of the *Arthrospira maxima* OF15 species. Moreover, Engin et al. [14] supplemented the basal Bold medium with 10% vinasse and obtained 0.97 day^{-1} growth of *Micractinium* sp. ME05. Thus, *Chlamydomonas* sp., *D. spinosus*, *H. pluvialis*, *S. quadricauda*, and *T. gracilis*, if submitted to cultivation processes such as manipulation or supplementation with vinasse, may present better results in terms of resistance and growth in vinasse.

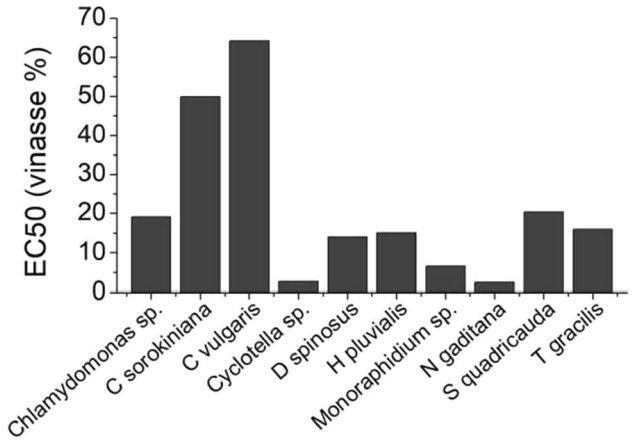

Fig. 2 EC50 values of centrifuged vinasse concentrations (%) that caused 50% decrease in growth rates in relation to the respective controls for the 10 microalgae prospected

However, for *N. gaditana* and the diatom *Cyclotella* sp., the reduction exceeded 50% of the control, demonstrating high vinasse inhibition in growth to these species. The 5% vinasse was not nutritionally limited to the microalgae as the growth rates for most tolerant species in 5% of vinasse were similar to the respective controls. Thus, the nutrients provided by the most diluted vinasse were enough for these microalgae. Regarding the EC50 values, there is a large variation between species, with values between 2.5 and 64.2%. This shows that the sensitivity to vinasse compounds is very variable among the species tested.

Viability and cell density

Table 3 reports the growth rates and final cell density of the eight selected species at their optimal vinasse concentrations in the second experimental stage. In vinasse, the species with the highest growth parameters were *C. sorokiniana*, *C. vulgaris* and *D. spinosus*, followed by *Chlamydomonas* sp., *S. quadricauda* and *Monoraphidium* sp. *T. gracilis*, and *H. pluvialis* had the lowest growth in vinasse among the tested species. The differences in the toxicities within and across algal species might be attributed to a number of explanations, including differences in compound uptake; differences in the binding pockets in the primary targets; differences in compounds elimination through modification or degradation and differences in active efflux pumps [32, 33]). The microalgae species that showed the best performance in cultivation with vinasse belong to the Chlorophyceae class. This result is in agreement with what was observed by Ji et al. [34] which identified Chlorophyceae (30.4%) as the main components of the class-level taxa present in municipal wastewater. Consequently, Chlorophyceae are the most functionally essential for COD, N, and P removal. Thus, they have greater tolerance to higher concentrations of vinasse. In addition, *Chlorella* sp. and *Desmodesmus* sp. are found naturally in freshwater, especially in nutrient-rich environments. They have already been widely cultivated as free cells and immobilized in industrial, domestic, and artificial wastewater, showing high cell viability, tolerance to pH and temperature variations, and similar specific growth rates [35, 36].

Higher growth parameters in vinasse compared with the respective controls were obtained for *Chlamydomonas* sp., *C. vulgaris*, and *S. quadricauda,* but lower growth rate was obtained for *Monoraphidium* sp. (Table 3). The other microalgae had growth parameters similar to their controls. The marine algal strains *N. gaditana* and *T. gracilis* were chosen in this study because they naturally grow in high conductivities in the marine habitat, closer to that of vinasse. However, the results presented showed that this did not help as the cells that adapted to the adverse conditions imposed by the waste. *N. gaditana* were inhibited in vinasse with no growth, despite the 1.3 day^{-1} growth rate in BG11 with salt.

Table 3 Mean values of growth rates (day^{-1}) and final cell densities (cells 10^5 mL^{-1}) for controls and cultures in optimum vinasse concentrations (%) for the microalgae

Species	Vinasse concentration	Growth rate (day^{-1})		Density (cells 10^{5} mL^{-1})	
		Control	Vinasse	Control	Vinasse
Chlamydomonas sp.	5%	0.84^{e}* (0.06)	0.97^{d}* (0.03)	6.62^{f}* (0.33)	14.40^{d}* (1.15)
C. sorokiniana	15%	1.49^{ab} (0.05)	1.54^{a} (0.02)	37.96^{b}* (0.71)	29.53^{c}* (1.01)
C. vulgaris	20%	1.21^{c}* (0.03)	1.41^{b}* (0.02)	12.23^{d}* (0.44)	16.57^{d}* (0.76)
D. spinosus	20%	1.23^{c} (0.04)	1.20^{c} (0.02)	14.90^{d} (0.70)	14.93^{d} (0.65)
H. pluvialis	5%	0.24^{g} (0.07)	0.34^{g} (0.06)	0.50^{h} (0.02)	0.64^{h} (0.07)
Monoraphidium sp.	5%	1.62^{a}* (0.05)	0.79^{e}* (0.04)	48.37^{a}* (0.67)	10.33^{e}* (0.70)
S. quadricauda	5%	0.75^{e} (0.04)	0.82^{e} (0.01)	5.02^{f}* (0.14)	8.84^{e}* (0.49)
T. gracilis	5%	0.64^{f} (0.04)	0.63^{f} (0.03)	3.93^{g} (0.19)	3.38^{g} (0.76)

The standard deviations from the mean are presented in parentheses. Different letters indicate values that differed significantly in each evaluated parameter ($p < 0.05$). Asterisk indicates values for which the growth in vinasse differed from the respective controls for each microalgal ($p < 0.05$)

Despite the high biotic growth capacity demonstrated in the control, the EC50 for this microalga was lower than 3% of vinasse, indicating a low tolerance to the waste, supported by the absence of growth in vinasse in 5%. *T. gracilis*, the other marine species tested, grew similarly to the control in 5% vinasse, with a higher tolerance to the waste, represented by EC50 values of 16% vinasse. According to Asma and Mathew [37], *T. gracilis* is an estuarine species and was able to reduce the concentration of organochlorine insecticides by up to 30% in aqueous solutions, confirming its ability to survive in medium with unfavorable organic compounds. Open ocean organisms are usually sensitive to toxic agents [36, 38], explaining why *N. gaditana* was less tolerant to the vinasse than *T. gracilis*. Despite the smaller final cell densities than BG11, the growth of *T. gracilis* in 5% centrifuged vinasse diluted with artificial seawater is commercially promising, as seawater is an abundant and a cheap resource.

Figure 3 shows the final total biovolumes and dry biomass (96 h). For *C. vulgaris*, the total biovolume in vinasse was 400% larger than in the control, while it was 200% greater for *Chlamydomonas* sp. and *D. spinosus*, 60% for *H. pluvialis*, 250% for *S. quadricauda* and 20% for *T. gracilis*. The most productive species in vinasse were *H. pluvialis*, *C. vulgaris*, *S. quadricauda*, and *T. gracilis*. On the other hand, the higher dry biomass in vinasse reached 300% compared with the control. Concerning this parameter, again the species *T. gracilis*, *H. pluvialis*, *C. vulgaris*, and *S. quadricauda* proved to be the most productive in vinasse.

Growth rates were similar for *H. pluvialis* and *S. quadricauda* in 5% vinasse and their respective controls ($p > 0.05$), but lower growth rates at 10% vinasse or above indicate that some component of the vinasse negatively affected their physiology. In accordance with the present results, recent research showed the ability of these species to grow in diluted vinasse, indicating their potential for waste remediation. Gollo et al. [39] grew *H. pluvialis* in 3% vinasse with 0.7% NaCl for 15 days. They argued that the microalgae growth enabled the use of the waste in plant irrigation because cytotoxicity, saline stress and phenolic compounds were reduced and no toxicity to the plants was observed. With the same species, Haque et al. [40] obtained a growth rate of 0.32 day^{-1} and 4.4 g L^{-1} of biomass in approximately 1.6% vinasse supplemented with 5% CO_2. In addition, the authors obtained a nutrient reduction of the waste, with a 67% decrease in total carbon and 91% in total nitrogen. In relation to *S. quadricauda*, Ramirez et al. [41] obtained 0.5 g L^{-1} in Guillard medium supplemented with 40% vinasse. Rocha et al. [42] obtained a 0.41 day^{-1} growth rate for *S. quadricauda* in synthetic medium, lower than the 0.75 day^{-1} growth rate we obtained in 5% vinasse. Therefore, the use of low concentrations of vinasse as a culture medium can be advantageous for certain microalgae compared with synthetic culture medium.

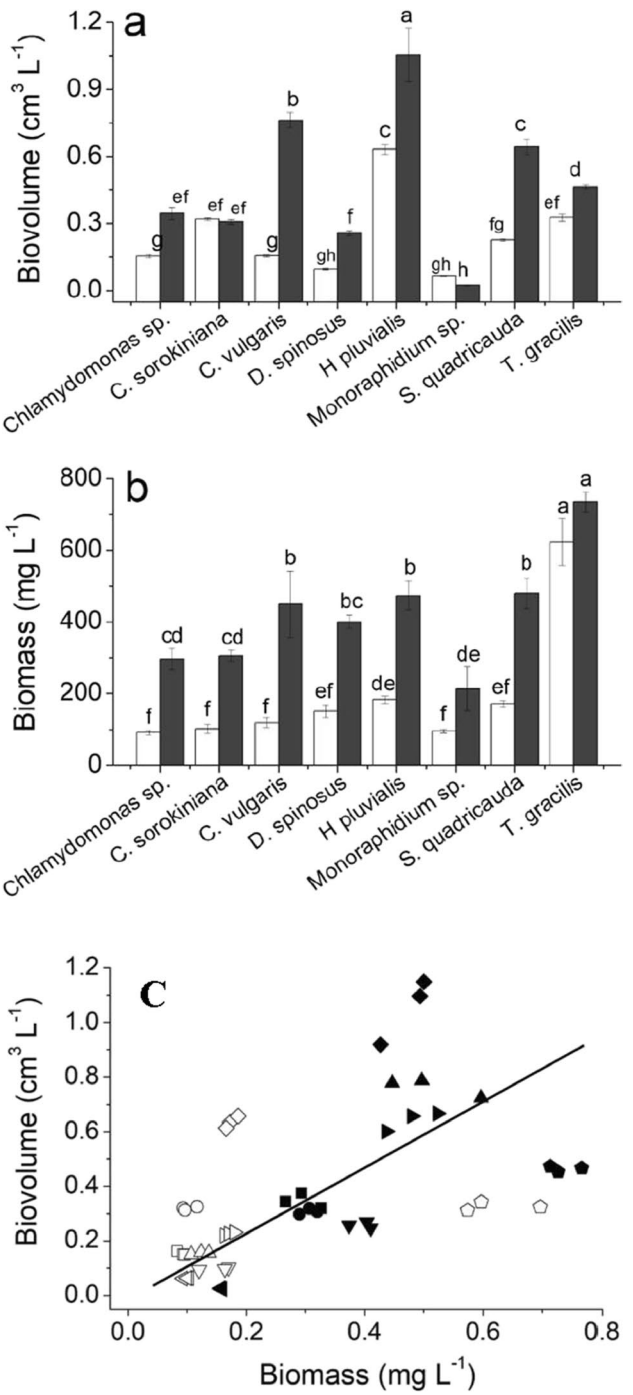

Fig. 3 Productivity parameters at 96 h cultures for biovolume of **a** total biovolume (cm^3 L^{-1}) and **b** dry biomass (mg L^{-1}) for the eight selected microalgae in BG11 controls and in cultures in vinasse (**c**). Total biovolume (cm^3 mL^{-1}) as function of dry biomass (mg L^{-1}) symbols apply for (/) *Chlamydomonas* sp., (/) *C sorokiniana*, (/) *C vulgaris*, (/) *D spinosus*, (/) *H pluvialis*, (/) *Monoraphidium* sp., (/) *S quadricauda* and (/) *T gracilis* Controls (AU; light bars and symbols) and at vinasse cultivation (AU; dark bars and symbols). Different letters indicate values that differ significantly from each other ($p \leq 005$) **H pluvialis* and *T gracilis* were not considered in Fig. 3c because the linear regression because of its complex life cycle and marine habitat respectively

The freshwater diatom *Cyclotella* sp., which has a low division rate, was highly sensitive and unable to grow in 5% vinasse, even though silica has been added. According to Lee [20], diatoms can have low growth rates because they need to synthesize silica frustules for the reproduction process. According to Saros and Anderson [43], the distribution of *Cyclotella* in aquatic environments is guided by the clarity of water and pH as this species does not tolerate acid environments. Thus, the presence of vinasse, which increased the load of dissolved organic matter in the medium, has also led to higher medium turbidity and lower pH, affecting the survival and reproduction of the microalgae.

In the second stage of the present study, the cell growth parameters for the microalgae at the respective optimal vinasse concentrations are, in most cases, statistically similar to the controls in BG11. When evaluating diverse media for the cultivation of *Botryococcus braunii*, Dayananda et al. [41] observed that the highest biomass and hydrocarbon productivities occurred in BG11. As this medium is rich in mineral nutrients [19] and widely used in large-scale cultures [42, 43], the results presented in vinasse are promising for larger scales.

Total biovolume refers only to microalgae, whereas dry biomass to the biological community, including microalgae, fungi and bacteria. Figure 3c reports the total biovolume as a function of dry biomass, showing a high correlation between these parameters. This indicates that the algae contributed significantly to the dry biomass. Therefore, among the microbiota analyzed, microalgae are the most representative organism in quantitative terms.

Biovolume is widely used in productivity measurement when it comes to phytoplankton communities, as addressed by Mandal et al. [44] and Sutherland et al. [45], but rarely applied to studies on population growth. Biovolume increase indicates biomass production in microalgae, leading to a decrease in the surface to volume ratio. This may have brought advantages to the enlarged organism in relation to contact surface with toxic compounds in vinasse [33, 34]. As expected from the increase in total biovolumes, the final dry biomasses were larger in vinasse than in controls. However, in addition to the higher algal biovolumes, contamination with bacteria and fungi in the vinasse cultures contributed to these increases in productivity values [4]. The present results are in accordance with Barrocal el al. [46], Marques et al. [10], Coca et al. [18], and Santana et al. [47] that reported higher productivity values for microalgae in vinasse, even though they did not mention the biological contaminants.

Another way of inferring the algal representability in the total biomass is by comparing the absorbance values at 570 nm and 684 nm, as presented in Fig. 4, which represent the total particulate and chlorophyll material, respectively [27, 28]. The absorbance at 570 and 684 nm in the controls were statistically similar for all species tested,

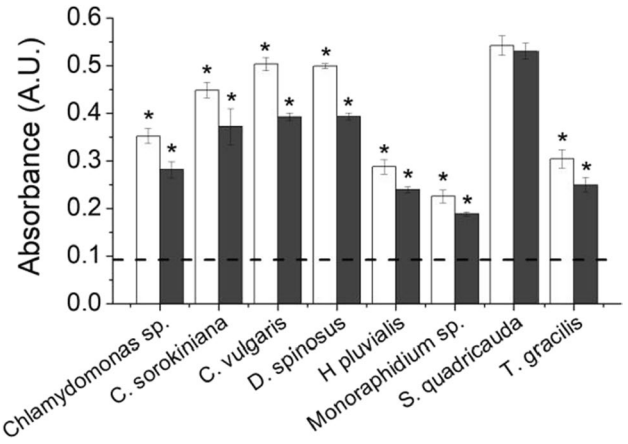

Fig. 4 Absorbances at 570 and at 684 nm in 96 h of absorbance at 570 nm (AU; light bars) and at 684 nm (AU; dark bars) in vinasse cultures. Error bars represent the standard deviation from the mean Asterisks indicate significant difference between values at 570 and at 684 nm ($p < 005$) The dashed line represents the mean absorbances at 570 and 684 nm in the controls

indicating that the biomass in BG11 basically consists of microalgae. Meanwhile, in vinasse treatments there are differences between the absorbance values at the two wavelengths at 96 h, pointing out the presence of contaminants in the biomass produced. However, this difference is significantly lower than the absolute absorbance values at 684 nm, indicating a dominance of microalgae, supporting the correlation between total biovolume and dry biomass. The largest differences between these parameters at 96 h occurred for *C. sorokiniana*, *C. vulgaris*, and *D. spinosus*, indicating maximum percentages of biological contaminants of 22% in relation to the biomass produced for each cultivated species.

Although total dry biomass alone does not represent the real microalgae biomass, in our results there is a great correlation between these two parameters. These high correlations could be shown by the similarity between the absorbance at 684 nm, that is relative to clorophyllate particles [28], and absorbance at 570 nm, which refers to the total particulate material [27]. In 96 h, the chlorophyll absorbance represented at least 78% of the total particulates (570 nm). The minimal percent contribution of chlorophyll to the total particulate occurred in *C. vulgaris* and *D. spinosus* cultures, for which the highest concentration of centrifuged vinasse was used. These higher concentrations can result in larger amounts of organic matter available to contaminating organisms [16]. In the case of low vinasse concentrations, lower organic load and heterotrophic microorganisms were introduced in the culture, and therefore microalgae were dominant in the biomass. Thus, studies on microalgae cultures in high concentrations of vinasse or other organic waste should consider the presence of contaminants when

reporting biomass productivity, as the risk of overestimation productivity and misinterpretation occurs.

Proteins and carbohydrates

The biomolecule proteins and carbohydrates and their ratio (P/C ratios) for the total biomass are reported in Table 4. The higher production of biomolecules in Chlamydomonas sp., *H. pluvialis*, *S. quadricauda*, and *T. gracilis* was coincident with the lowest growth rates among the microalgae tested. This can be explained by the higher total biovolume that these species also presented, confirming that growth rate alone may not be a good parameter of productivity, particularly in waste. Different from other literature studies of microalgae in vinasse that report the accumulation of storage molecules as lipids and carbohydrates [12, 36], in our study proteins accumulated, not carbohydrates. This is confirmed by the protein/carbohydrate ratio (P/C) that was around 1.0 for the controls and greater than 2.0 for the vinasse cultures.

For total proteins, the highest yields in vinasse were in *D. spinosus*, *S. quadricauda*, *Chlamydomonas* sp., *C. vulgaris*, and *H. pluvialis*, followed by *C. sorokiniana*, *Monoraphidium* sp., and *T. gracilis*. For carbohydrates, the most productive species in vinasse were *H. pluvialis*, *S. quadricauda*, *Chlamyddomonas* sp., and *T. gracilis*, followed by *D. spinosus*, *C. sorokiniana*, *C. vulgaris*, and *Monoraphidium* sp. Vinasse stimulated increases in the production of proteins for all the microalgae, but did not usually interfere with carbohydrates. Consequently, the P/C ratios had significant increases in the diluted centrifuged vinasse over the BG11 controls.

According to Rocha et al. [42], ratios greater than 1.0 indicate that microalgae are physiologically healthy as storage biomolecules such as lipids and carbohydrates are accumulated under stressing conditions. In the case of vinasse cultures, proteins accumulated. Coca et al. [18] also observed this predominance of proteins in *Spirulina platensis* biomass generated in vinasse; Santos et al. [48] observed the same for *Spirulina maxima*. Considering that in microalgae, proteins can be structural components, enzymes, pigments, and complexing agents, its increase in vinasse may be related to both structural, because of increase in cell biovolume, and toxicity-related proteins, used to overcome the possible negative effects of the vinasse. Besides that, the presence of yeast may have contributed to the higher protein values in vinasse cultures compared to controls. According to Cochrane [47] and Raven et al. [48], yeasts and other fungi are rich in proteins. In any case, protein richness and moderate amounts of carbohydrates in the biomass generated in vinasse may be interesting for animal feed and fish farming, as presented in Borowitzka [3].

In general, we showed that different microalgae had different tolerance to vinasse, highlighting the importance of systematic investigations related to the prospection of microalgae in waste such as vinasse. Environmentally, this research points to an important warning, especially because microalgae constitute the base of trophic chains in aquatic ecosystems [49]. Considering how different the physiological responses were of the microalgae species we tested, with different tolerances to vinasse, contamination of aquatic ecosystems by this waste, even diluted, can imbalance species abundance, affecting the phytoplankton biodiversity. Consequently, this will end up disrupting the equilibrium of food chains. According to Silva et al. [1], the application of vinasse onto soils of sugarcane crops as fertilization practice can contaminate underground and surface water bodies. Therefore, it is important that other uses for vinasse should be developed, thus preventing environmental contamination, as part of the composition of microalgae culture media.

Table 4 Biomolecules in total biomass: total proteins (mg L^{-1}), total carbohydrates (mg L^{-1}) and proteins/carbohydrates ratios (P/C ratios) in controls and optimal vinasse concentrations for the total biomass

Species	Proteins (mg L^{-1})		Carbohydrates (mg L^{-1})		P/C Ratio	
	Control	Vinasse	Control	Vinasse	Control	Vinasse
Chlamydomonas sp.	10.23^{cd}* (0.36)	46.85^{ab}* (0.94)	10.94^{cd} (0.92)	11.90^{cd} (0.42)	0.94^{f}* (0.05)	3.94^{c}* (0.21)
C. sorokiniana	5.56^{e}* (0.22)	37.42^{b}* (1.95	5.83^{f}* (0.63)	7.21^{ef}* (0.47)	0.96^{f}* (0.10)	5.20^{*b}* (0.31)
C. vulgaris	5.65^{e}* (0.97)	45.98^{ab}* (2.09)	5.89^{f} (0.31)	6.67^{f} (0.86)	0.97^{f}* (0.21)	6.99^{a}* (1.16)
D. spinosus	4.81^{e}* (0.67)	49.65^{a}* (1.12)	7.37^{ef} (0.76)	7.99^{ef} (0.95)	0.66^{f}* (0.15)	6.23^{ab}* (0.54)
H. pluvialis	19.24^{c}* (4.62)	41.62^{ab}* (5.06)	18.64^{a} (1.01)	18.77^{a} (0.82)	1.04^{f}* (0.29)	2.22^{de}* (0.26)
Monoraphidium sp.	4.14^{e}* (0.45)	22.31^{c}* (2.39)	5.30^{f}* (0.64)	6.47^{f}* (0.52)	0.78^{f}* (0.06)	3.45^{c}* (0.38)
S. quadricauda	4.94^{e}* (0.63)	48.13^{ab}* (3.57)	12.99^{bc}* (0.79)	14.57^{b}* (0.28)	0.38^{f}* (0.03)	3.30^{cd}* (0.26)
T. gracilis	8.07^{d}* (1.25)	18.66^{c}* (0.40)	9.38^{de} (1.31)	10.11^{de} (0.52)	0.86^{f}* (0.09)	1.85^{ef}* (0.08)

The standard deviations from the mean are presented in parentheses. Different letters indicate values that differed significantly in each evaluated parameter ($p < 0.05$). Asterisk indicates values for which the growth in vinasse differed from the respective controls for each microalgal ($p < 0.05$)

Conclusions

The bioprospection of the analyzed strains showed that microalgal growth rates in vinasse were similar to cultures in the nutrient rich BG11 synthetic medium, but algal productivities were higher in the waste as vinasse stimulated increases in total microalgae biovolumes. The microalgae *H. pluvialis*, *S. quadricauda*, and *T. gracilis* had the lowest growth rates among the tested species but had the highest total biovolume. *Chlamydomonas* sp., which also presented high productivity, had the advantage of growing in dilute vinasse with less need for pH adjustment. *T. gracilis* stands out because it grows in vinasse dilution with seawater, an abundant resource, and makes it difficult for biological contaminants to develop. *C. sorokiniana*, *C. vulgaris*, and *D. spinosus* were the most tolerant organisms to vinasse, grown in 15–20% of the waste. Despite the need to dilute the vinasse for microalgae growth, it guarantees economy with nutrients of synthetic media and has a higher yield of biomass than in BG11, with less contamination than when vinasse is used without dilution. Allied to these beneficial effects, the microalgae *Chlamydomonas* sp.; *D. spinosus*, *S. quadricauda*, and *H. pluvialis* showed high synthesis of total proteins that may have the contribution of biological contaminants. Thus, bioprospecting showed species with high growth rates and biomass production, as well as lower biomass production but better biomolecular content.

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