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# The physiology of *Chlorella vulgaris* grown in conventional and biodigested treated vinasses

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ABSTRACT

Conventional and biodigested vinasses are organic and nutrient rich residues from the sugar cane alcohol industry. Their disposal in running waters and soils are controlled due to their eutrophication potential. Using it as algal culture medium, lower costs and residue remediation can be achieved. Here, we report on the physiology of *Chlorella vulgaris* grown in 60% conventional and 80% biodigested treated vinasses in 6 days experiments. Cell densities, chlorophyll *a* concentrations and pulse amplitude modulated (PAM) fluorometry were used for culture monitoring. Maximum and operational quantum yields, photochemical (qP) and non-photochemical quenchings (qN and NPQ) and rapid light response curves were determined and efficiency of light use (α) and light saturation ($I_K$) obtained for all treatments. The results showed that *C. vulgaris* grew better in vinasse (1.5–1.6 $d^{-1}$) than in controls (0.9–1.2 $d^{-1}$). PAM fluorometry showed that, despite the increased algal growth in vinasses, photosynthesis was higher in controls than in the residues, supporting that *C. vulgaris* used organic carbon as energy source. We suggest that the high competitiveness of the microalgae was due to its ability to grow mixotrophically in the residue even though heterotrophic contaminants were present. A reduction of the vinasses eutrophication potential was obtained after the microorganisms' growth. This study is a contribution to the knowledge of microalgae photosynthetic physiology in organic rich residues, information that can help improve algal biomass production in residues at the same time that performs its remediation.

## 1. Introduction

Microalgae are photosynthetic organisms that can present several metabolic pathways for carbon and energy acquisition, which can be summarized as photoautotrophy, heterotrophy and mixotrophy. Although literature results show that the majority of microalgae can have mixotrophic metabolism [1–4], this has not been investigated using techniques that measure its photosynthetic performance and incorporation of organic carbon at the same time.

Photosynthesis can be divided into photochemical and biochemical steps [5], the light dependent and independent reactions, respectively. In the photochemical step, light energy hits photosystem II (PSII) and photosystem I (PSI), where it is captured and transferred to the pair of chlorophyll *a* in the reaction centers. From there, excited electrons begin their way through the electron transport chains [6]. In the light dependent stage, $O_2$, ATP and NADPH are generated, with the last two molecules being used to form glucose in the light independent reactions. The efficiency with which the light reactions occur can be measured by PAM fluorescence, a technique that can tell about the algal physiological status. Information about the energy that goes to the photochemistry step of photosynthesis or the fraction that is lost due to stressful situations are easily detected. According to Lombardi and Maldonado [7], problems in photosystem II are identified by this technique. So, information regarding its photosynthetic condition and health status can be obtained.

Vinasse is a liquid residue from sugar cane industry that is turbid, fetid and rich in organic matter and mineral elements and it is produced in amounts as high as 12 L vinasse/L alcohol. Due to its high pollutant potential [8], vinasse disposal in water bodies is unsafe, forcing alternative uses and/or treatments, as its biodigestion (vinasse anaerobic fermentation). This process generates biogas, which is used by the sugar industry as energy source, but the polluting potential of the resulting biodigested vinasse remains [9]. In the present text, vinasse is classified as conventional (raw vinasse) or biodigested (fermented vinasse).

Due to vinasse mineral richness and organic content, a possible application is its use as a culture medium for microalgae, which is the target of this study. Thus, vinasse is a residue that could support mixotrophic metabolism in microalgae, a situation where illumination

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would be less determinant and the organism could use the dissolved organic matter in the vinasse as carbon source. Furthermore, using a residue as culture medium, microalgae large scale production costs could be reduced. However, despite the vinasse's nutritional value, it is a challenge to grow photosynthetic or mixotrophic microalgae having it as culture medium. Its dark brown color decreases light penetration, the high nutrient content increases its conductivity (possibly causing osmotic problems) and its contamination by heterotrophic microbes increases competition for resources [4]. This sum of factors makes either conventional (raw vinasse) or biodigested vinasses inhibitory to microalgae, forcing the residue dilution and/or previous treatments for its use as algae culture medium.

Candido and Lombardi [4] proposed a treatment for sugar cane vinasse that was based in its filtration through smectite clay and activated carbon. The authors showed that *Chlorella vulgaris* had optimum growth in vinasses concentrations as high as 60% conventional and 80% biodigested. As a continuation from the previous study, in this research we cultivated *C. vulgaris* in previously filtered 60% conventional and 80% biodigested vinasses to evaluate the physiology and possible mixotrophic metabolism of the microalgae. This was performed through measurements of the photosynthetic activity using PAM fluorescence for the determination of rapid light response curves, quenchings, and photosynthetic quantum yields, and chemical analyses of the dissolved organic matter and nutrient content in the medium.

## 2. Material and methods

The conventional (cv) and biodigested (bv) vinasses used in this study were donated by São Martinho Plant located in Pradópolis (SP, Brazil) in the beginning of the sugar cane harvest. It was stored in 1 L capacity plastic bottles in a freezer (− 8 °C) until use. Previous to its use, the vinasses were defrosted and filtered through smectite clay and, subsequently, in activated carbon, reducing the amount of particulate material, clarifying and raising its pH [4,10,11].

The treated vinasses were used as culture medium after dilution with autoclaved distilled water (121 °C, 1 atm, 20 min). The vinasses were used in the concentrations of 60% for the conventional and 80% for the biodigested, which according to previous investigation [4], resulted in the best growing conditions for *C. vulgaris*. Vinasses were not sterilized, so biological contaminants were present.

The strain of *Chlorella vulgaris* used was isolated from a sewage treatment plant and is maintained in the Laboratory of Algae Biotechnology (Federal University of São Carlos, Brazil) under the reference LBA 01. We performed a control with LC Oligo medium [12], but to compare our results with a well known medium that supports high cell densities, cultures were also performed in BG11 culture medium [13]. In the BG11 just cell density and growth rate were evaluated. Algal cultures were performed in 250 mL capacity cell tissue culture bottles with 150 mL of medium, had initial pH adjusted to 6.8–7.0 and were inoculated with exponentially growing stock cultures at final cell density of $5 \cdot 10^4$ cells $mL^{-1}$. Prior to the cell inoculation in the vinasse treatments, microalgae were acclimated during 72 h in the same composition of each treatment.

Three experimental replicates were performed and cultures were kept under controlled temperature (24 ± 2 °C), light:dark cycle (12:12 h) and internal irradiance (150 μmol photons $m^{-2} s^{-1}$). The cultures were positioned at different distances from the light source depending on their turbidity, so that cells received the same light intensity for all treatments independent of the vinasse concentration. Cultures lasted 6 days and were sampled daily for algal cell density (cell $mL^{-1}$), chlorophyll *a* concentration (mg $L^{-1}$) and heterotrophic colony counts. The cell density was obtained by counting *C. vulgaris* (4% formaldehyde preserved cells) in a Fuchs-Rosenthal chamber under optical microscope (Nikon Eclipse model E200, Japan). Population growth rates were obtained by linear regression of the natural logarithm of cell density in the exponential growth phase, whose slope corresponds to the specific growth rate. Chlorophyll *a* concentration in the microalgae cells was determined daily through in vivo fluorescence in a Trilogy fluorimeter (Turner Designs - USA). Fluorescence was converted to chlorophyll *a* concentration (mg $L^{-1}$) by means of a linear regression equation obtained after the instrument calibration (chlorophyll *a* concentration against fluorescence). Dividing the obtained chlorophyll *a* concentration by the cell density, values of chlorophyll *a* per cell were obtained.

The heterotrophic contaminants (bacteria, yeast and other fungi) in the cultures were monitored daily through colony formation units that were counted on plates using plate count agar media (PDA; OXOID, U.K.) and Sabouraud Dextrose Agar (HiMedia; India). The heterotrophic microorganisms' counts were considered for intervals between 30 and 300 colonies of bacteria and between 20 and 200 fungi colonies per plate, situations in which colonies viewing were better and statistical errors are lower. Each sample was diluted 4 times and plated daily. Eppendorf tubes containing 900 μL of phosphate-buffered saline (PBS) solution to which 100 μL of the sample for the first dilution ($10^{-1}$) was added. Throughout the culturing period, cultures dilutions up to $10^{-6}$ final concentration were used.

The platings were done under filtered air using a Drigalski handle for scattering, and a sterilizer (Marconi MA1202 – CT, Brazil) at 200 °C was used for the Drigalski handle sterilization between samples. The plates were incubated at 32 ± 2 °C in the dark. For the bacteria, PCA plates were incubated for 48 h, and for the yeast and filamentous fungi, Sabouraud plates were incubated for 120 h, after which the colonies were counted (CFU).

Total organic carbon (TOC) and total organic nitrogen (TON) content in the vinasses were determined at the beginning and end of experiments (Shimadzu model ASI-L, Japan). For theses determinations, the removal of the biological material from the vinasse was performed through centrifugation (10 min; 4400 rpm; 20 °C), the pellet was discarded and the supernatant followed to TOC and TON determinations. Chemical characterization of the 60 and 80% vinasses according to Brazilian requirements, before and after experimentation, was performed and the methodology is listed in Table 1.

For the photochemistry of photosynthesis, a Phyto PAM fluorimeter (Walz - Germany) was used. The maximum ($\phi_m$) and operational ($\phi_{m'}$) photosynthetic efficiency and energy decays (fluorescence quenchings) qP, qN and NPQ were determined in both the exponential and stationary phases of microalgae growth. According to literature [14], the photochemical quenching (qP) indicates the proportion of light energy that is used in photosynthetic processes; the non-photochemical quenching qN is related to all fractions of light energy not used in photosynthesis, while the non-photochemical quenching NPQ is mostly related to energy dissipated as heat. The values of Fo, Fm and Fv were obtained directly from the instrument in dark adapted cells, and F, Fo′ and Fm′ in light adapted cells. The photosynthetic variables were obtained according to the equations presented below [15,7].

$$Fo' = Fo/((Fv/Fm) + (Fo/Fm')) \quad (1)$$

$$qP = 1 - (Fm' - Fo)/(Fm' - Fo') \quad (2)$$

**Table 1**
Methods of chemical characterization. Methods used for the chemical characterization of the 60% conventional (cv) and 80% biodigested (bv) treated vinasses.

| Variable | Methods |
|---|---|
| Anions | USEPA300.1 rev 1:1997 - POPDAM054 vs. 08:2013 |
| Amoniacal nitrogen | POPDAM016 vs. 14:2013 - SMEWW 22ª Ed 2012 - Method 4500-NH3D |
| Kjeldahl nitrogen | POPDAM107 vs. 06:2013 - SMEWW 22ª Ed 2012 – Method 4500-Norg B |
| Total metals | SMEWW 22ª Ed 2012 - Method 3030E USEPA 6010 C - rev.03:2007 POPDAM060 |