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COD and nitrogen removal from sugarcane vinasse by heterotrophic green algae *Desmodesmus* sp.

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ABSTRACT

Vinasse is the main wastewater from the ethanol fermentation–distillation process, generated in large volumes during industrial sugarcane processing. *Desmodesmus* is a green algae genus with recognized ability to treat wastewater containing organic matter and to consume nutrients under heterotrophic growth conditions. Thus, the aim of this research was to evaluate *Desmodesmus* sp. growth in sugarcane vinasse. Results indicated slight elevation of pH, low oxygen, and low carbon dioxide consumption. Nitrogen and chemical oxygen demand (COD) removal were 52.1 and 36.2%, respectively. Specific growth rate of 0.15 h^{-1} and high yield of COD to biomass at first hour (0.5 mg mg^{-1}) suggest the feasibility of biomass production of this green algae in sugarcane vinasse.

Keywords: Vinasse; Green algae; Wastewater treatment; *Desmodesmus*

1. Introduction

Vinasse is the major effluent from the ethanol fermentation–distillation process. It has acid pH, which promotes its high corrosive characteristic, and high concentration of organic matter, potassium, and nitrogen, being capable of superficial water eutrophication and soil salinization when discharged in an arbitrary way [1,2]. Some industrial uses have already been studied like biodigestion, burn, thermal and chemical concentration and fertigation [3]. Nowadays, the fertigation alternative is widely used, since potassium is able to improve sugarcane biomass yield [2]. However, in these processes, a large amount of organic matter is discarded without being used, which seems that a good opportunity for biomass production is being lost [4,5].

Scenedesmus shows high cellular viability in domestic effluents tolerating high variations in temperature and pH parameters. Martínez et al. [6] reported specific growth rate of 0.044 h^{-1} in a secondary treatment effluent, where this microalgae was able to remove almost all phosphorus and nitrogen from the effluent. In a similar experiment, Li et al. [7] reported specific growth rate of 0.034 h^{-1} for *Scenedesmus* sp. LX1 grown in synthetic effluent using ammonia as nitrogen source. Sacristán de Alva et al. [8] reported phosphorous and nitrogen removal of 66 and 94%, respectively, by *Scenedesmus accutus* from domestic effluent at 18 h cultivation, obtaining a biomass with 28% of lipid content. In a piggery effluent, Abou-Shanab et al. [9] obtained 60% of phosphorous and nitrogen removal after 20 d cultivation of *Scenedesmus*

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obliquus, reporting a 70% removal of calcium after this period. In autotrophic cultivations, most nitrogen removal is caused by pH alkalinization, which gives microalgae an important role in secondary effluent treatment [10]. Accordingly, Ji et al. [11] obtained 99% removal of nutrients after 96 h autotrophic cultivation of *S. obliquus* reporting a specific growth speed of 0.047 h^{-1}. Similar level was observed by Samorì et al. [12] using *Desmodesmus communis* and by Martínez et al. [6] using *S. obliquus*.

Desmodesmus, a recently created genus from *Scenedesmus* accordingly to An et al. [13] due to its spiny formations and genotypic differences, has members with close capacity of consuming wastewater nutrient under heterotrophic conditions. Bastos et al. [14], using vinasse as growth media for *Desmodesmus* sp., reported specific growth rate of 0.096 h^{-1} and pointed that these results are promising for high added value biomass generation in agroindustrial effluents. Biomass generated with microalgae production using industrial effluents as substrate seems to be an alternative for expensive wastewater treatment, since it can be used in aquaculture as a valuable feed ingredient [15]. Thus, the aim of this research was to evaluate chemical oxygen demand (COD), nitrogen removal, and growth of *Desmodesmus* sp. in sugarcane vinasse.

2. Materials and methods

2.1. Vinasse

Vinasse was collected from a sugarcane processing industry in the city of Araras, São Paulo, Brazil, directly after the ethanol distillation at 96°C, fractionated in 2 L bottles at approximately 80°C in the laboratory, and frozen until use.

2.2. Inoculum and cultivation

Green microalgae (*Chlorophytae*) *Desmodesmus* sp. was maintained in culture bank in modified BGN media [16] with the following composition: 30 mg L^{-1} K_2HPO_4, 75 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 36 mg L^{-1} $CaCl_2{\cdot}2H_2O$, 6 mg L^{-1} ferric ammonium citrate, 1 mg L^{-1} Na_2 EDTA, 6 mg L^{-1} citric acid, 20 mg L^{-1} Na_2CO_3, 1.5 mg L^{-1} $NaNO_3$, 72 mg L^{-1} NaCl, 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2{\cdot}4H_2O$, 0.22 mg L^{-1} $ZnSO_4{\cdot}7H_2O$, 0.39 mg L^{-1} $NaMoO_4{\cdot}2H_2O$, 0.079 mg L^{-1} $CuSO_4{\cdot}5H_2O$, and 0.04 mg L^{-1} $CoCl_2{\cdot}6H_2O$.

Desmodesmus sp. inoculum was grown autotrophically in the bioreactor using BGN media with pH 7.5, at 25°C, aeration of 1VVM, 3 xg mechanic agitation, and photo flux of 45 μmol m^{-2} s^{-1} until 1 g L^{-1} biomass concentration. After that, biomass was centrifuged at 1,275 *g* and inoculated in vinasse.

Heterotrophic cultivation of *Desmodesmus* sp. was carried out in stirred batch bioreactor (covered to prevent light incidence) with vinasse at pH 7.0, 25°C, 1 VVM, and 3 g of stirrer speed. Parameters such as pH, dissolved oxygen, and carbon dioxide were monitored and recorded every 30 min. Samples from the bioreactor were collected every 3 h for 30 h.

2.3. Analysis

COD of vinasse was obtained by the dichromate method, according to APHA [17], using an acid solution made of sulfuric acid and dichromate for the samples digestion at 150°C for 2 h in an HACH® digestor block. Digested samples were cooled to ambient temperature and the absorbance read at 600 nm in HACH® spectrophotometer using 4 mL glass cuvettes and calibration curve with biftalate solution.

Total nitrogen (TN) of vinasse was made using TN Test'N Tube™ HACH® vials kit. In the first step, samples were digested in an alkaline media with persulfate for 30 min at 105°C in HACH® digestor block, similar to COD, cooled at ambient temperature. A bisulfate reagent was poured into the vial and mixed followed by a reaction time of 3 min after which an indicator was added to react for 2 min. For the final step, samples of 2 mL from the digested tubes were added to acid reagent tubes for the color formation and absorbance reading in a HACH® spectrophotometer at 410 nm.

Suspended solids for estimation of biomass was performed by vacuum filtration using a Millipore® filtration apparatus with 0.22 μm Sartorius® cellulose acetate membranes previously dried at 105°C for 60 min and weighted. Filtration was performed with 5 mL samples and a blank from the suspended solids of vinasse was made before the inoculation of *Desmodemsus* sp. in the bioreactor. After filtration, membranes were dried at 105°C until constant mass measurement and the dry weight of suspended solids was obtained by weighting the difference between initial and after filtration weight of the membranes.

2.3.1. Green algae and bacteria cell counting

Desmodesmus sp. cells were counted using improved Neubauer chamber, counting the corner squares and the central one. Average of the counted values was performed and multiplied by the dilution factor and 10^5 for the cells per mL concentration.

Bacterial colony-forming units (CFU) were measured using Petrifilm Aqua™ from 3 M® applying 1 mL of the samples per plate and incubated for 48 h at 37°C. In this method, entire plate area is counted, the value is multiplied by the dilution factor and the result is expressed in CFU.

2.3.2. Kinetic analysis

Percentage efficiency of substrate removal from COD or TN was determined by following equation:

$$E - S = \frac{s_0 - s}{s_0} \times 100 \quad (1)$$

From the biomass concentration, COD and TN were calculated by the specific growth rate and consumption of COD and TN, according to the following equations:

$$\mu_x = \frac{1}{X}\frac{\mathrm{d}(X)}{\mathrm{d}t} \quad (2)$$

$$q_{\mathrm{COD}} = \frac{1}{X}\frac{\mathrm{d}(\mathrm{COD})}{\mathrm{d}t} \quad (3)$$

$$q_{\mathrm{TN}} = \frac{1}{X}\frac{\mathrm{d}(\mathrm{TN})}{\mathrm{d}t} \quad (4)$$

Biomass productivity was calculated according to the following equation:

$$P = \frac{X - X_0}{t} \quad (5)$$

In the log phase, specific growth rate presents the maximum value, calculated by following equation:

$$\mu_{\max} = \frac{\ln(X_2 - X_1)}{t_2 - t_1} \quad (6)$$

Yields of COD and TN to biomass were calculated by ratio of growth and consumption rates, i.e. from the slope of curve biomass vs. substrates (Eq. 7):

$$Y_{X/S} = -\frac{\mathrm{d}(X)}{\mathrm{d}(S)} \quad (7)$$

3. Results and discussion

Initial chemical composition and pH of vinasse are shown in Table 1. The data show that there is a need for adjustment of pH for the cultivation of green algae since pH of propagation medium BGN is 7.6. Another important point is the C/N ratio of vinasse sample around 20. This value calculated from the ratio in COD and TN is in the optimum range for growing these micro-organisms [18].

Fig. 1 presents dissolved oxygen and carbon dioxide profiles in vinasse during the growth of *Desmodesmus*. sp. As shown, oxygen consumption increased until the 15th hour of cultivation, stabilizing at 20% of the oxygen pumped into reactor until the end of the experiment. In this period, oxygen consumption occurred at a constant rate (non-limiting) of 0.186 mg L^{-1} h^{-1}, following zero-order kinetics. On the other hand, the production of carbon dioxide remained constant from 6 to 21 h at a rate of 2,608 mg L^{-1} h^{-1}.

These results demonstrate that oxygen consumption was high inside the bioreactor considering that, with 1 VVM aeration, pH did not acidify during the experiment (as expected for a controlled aerobic system), which may indicate that 1VVM aeration was higher than the cultivation process needed. Similar to the low oxygen consumption observation, the low carbon dioxide accumulation in the bioreactor may be linked to the high aeration used, i.e. 1 VVM, and may support that this was excessive in terms of biological demand. Also, the carbon dioxide production in bioreactor, related to organic matter consumption by the heterotrophic microorganisms, means that even being a stressing media, vinasse can be treated biologically without dilution.

pH in the bioreactor, in contrast to that described in the literature, increased from 7.7 to almost 8.5 during 30 h. A faster increase was observed in the first 10 h and continued in a slower manner for the last 20 h as shown in Fig. 2.

Increase in pH is related to the active transportation of some specific carbohydrates through the cell membrane by the symport system. It promotes media alkalinization by a proton uptake accompanied by carbohydrate transportation, lowering the free H^+ concentration in the media and so, misbalancing pH [19,20]. This suggests that vinasse presents specific organic molecules and *Desmodesmus* sp. was able to consume organic material under dark conditions.

TN consumption during cultivation occurred in an almost linear uptake after 9 h, reaching 52% at 30 h, as shown in Fig. 3.

It is likely that part of nitrogen depletion was caused by pH alkalinization and ammonia volatilization supported by aeration. Despite this, nitrogen concentration only started to decrease after 9 h of cultivation and after pH was already at 8.1 and, as

Table 1
Vinasse composition

pH	COD (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})	Total solids (mg L^{-1})
3.5	27,100	1,420	2.61	26,220

Notes: COD: chemical oxygen demand; TN: total nitrogen; TP: total phosphorus.

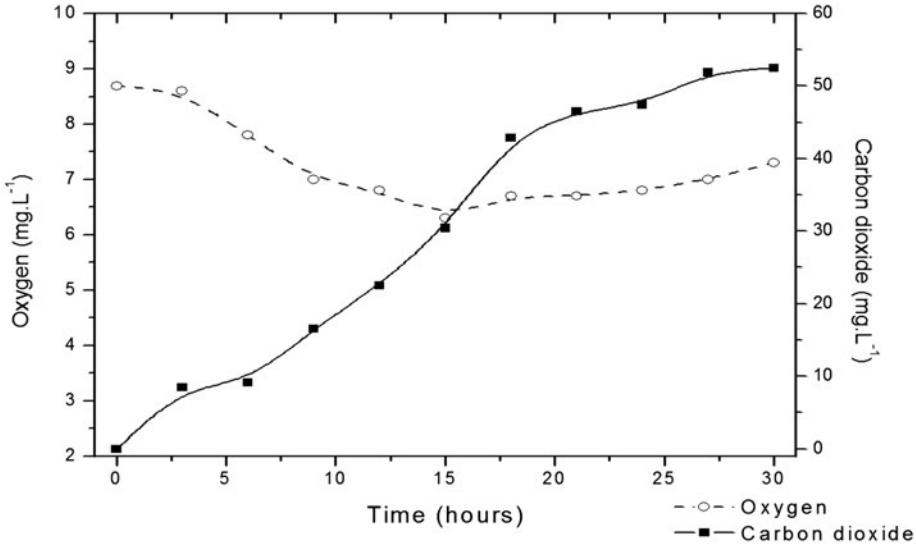

Fig. 1. Oxygen consumption and CO_2 production during the growth of *Desmodesmus* sp. in sugarcane vinasse.

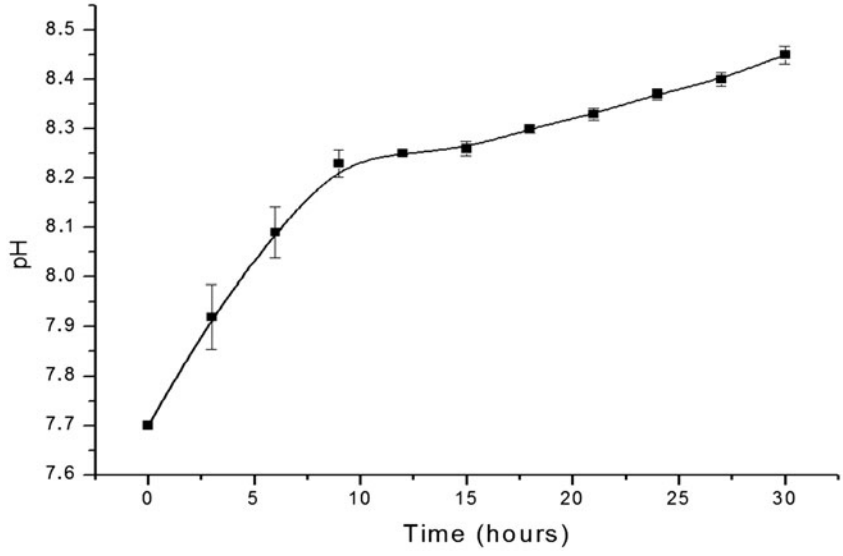

Fig. 2. pH evolution during the growth of *Desmodesmus* sp. in sugarcane vinasse.

shown in the biomass production topic, nitrogen concentration in the media started to decrease when the log phase of biomass growth was reached, indicating that its sequestration from the media did not happen by physical–chemical ways but by biological consumption.

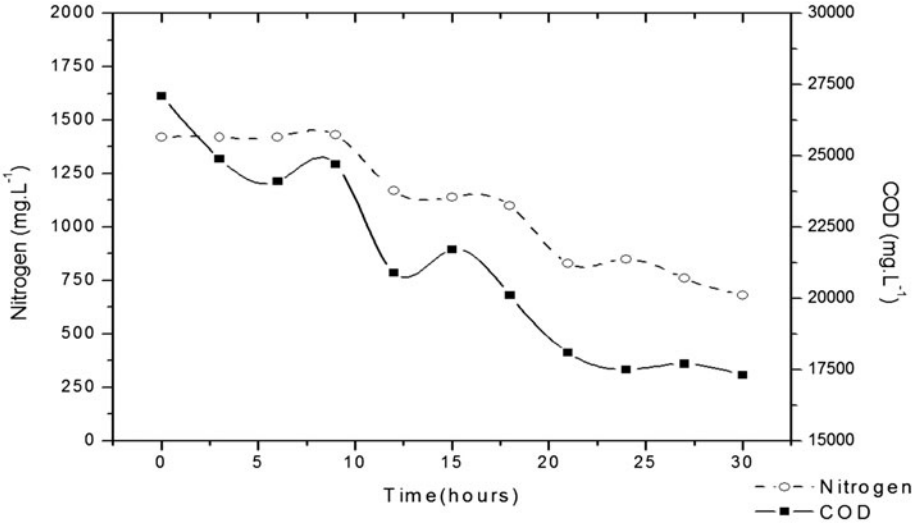

Fig. 3. Nitrogen and COD consumption by *Desmodesmus* sp. in sugarcane vinasse at 30 h experiment.

Table 2
Kinetic parameters

Parameter	Value
$\mu_{máx}$ (h^{-1})	0.15 ($R^2 = 0.87$)
q_{COD} (mg L^{-1} h^{-1})	342.8 ($R^2 = 0.95$)
q_{TN} (mg L^{-1} h^{-1})	32.88 ($R^2 = 0.95$)
E—COD (%)	36.2 (30 h)
E—TN (%)	52.1 (30 h)

Notes: $\mu_{máx}$: maximum specific growth rate; q_{COD}: COD consumption specific rate; q_{TN}: TN consumption specific rate; *E*—COD: maximum COD removal; *E*—TN: maximum TN removal; R^2: coefficient of correlation for linear fit of growth, COD, and TN curves.

According to Table 2, which presents the kinetic parameters as maximum specific growth rate of *Desmodesmus* sp. in vinasse, consumption rates, and maximum removals of COD and TN, nitrogen consumption in bioreactor was 32.88 mg L^{-1} h^{-1}, with maximum removal of 52% at 30 h, which agrees with the results of nitrogen consumption by *S. obliquus* in autotrophic cultivation using artificial wastewater shown by Nuñes et al. COD in the media decreased in an exponential trend, reaching 36.2% in the 30 h cultivation, as shown in Fig. 3, with consumption rate of 342.8 mg L^{-1} h^{-1}.

The faster consumption period was in the first 20 h and stabilized after that. This may be due to the quality of the rest of organic matter present after that. A possible explanation is that in the first 20 h, low molecular weight organic compounds supported biomass growth, and after its consumption, only high molecular weight organic compounds were left. As this is a hard consumption substrate, COD depletion slowed down, accompanied by a shock in the biomass that stabilized the growth after that.

The log phase of biomass growth started at the hour 9 lasting until hour 18 and biomass implementation in the bioreactor after 30 h cultivation with maximum specific growth rate of 0.15 h^{-1} (Fig. 4).

The carbon dioxide production, oxygen consumption, COD, and TN concentration presented higher variations after the log phase of biomass growth was reached, confirming that biomass was not only surviving the stressing media but growing in it, meaning that the C/N ratio near to 20 for sugarcane vinasse is suitable for the growth of such micro-organisms even under dark conditions [18]. In fact, Xing et al. [21] found that variations in the C/N ratio in cultivation medium resulted in different rates of incorporation of carbon and nitrogen in the cells.

Thus, a possible explanation for the shortness of the log phase is the misbalance of C/N ratio as nitrogen was consumed faster than carbon, which

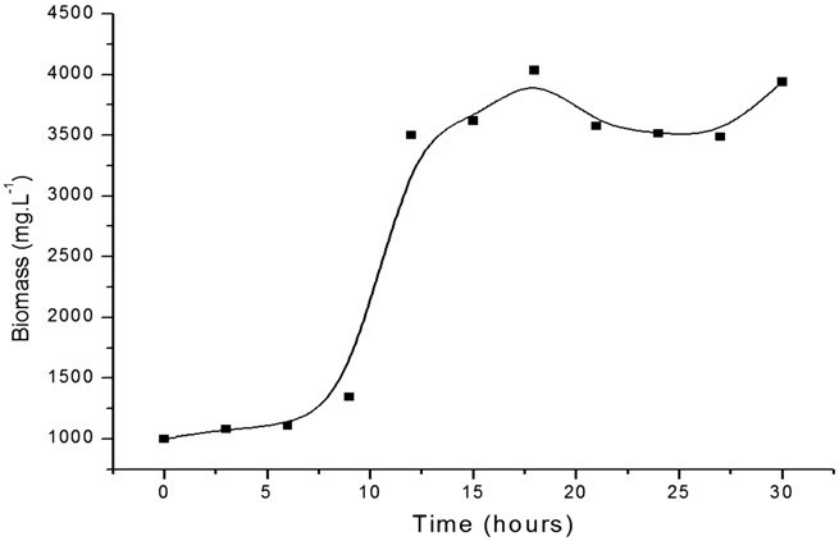

Fig. 4. Biomass concentration profile during the growth of *Desmodesmus* sp. in sugarcane vinasse.

may cause stressing situation in organic systems. Nevertheless, other possible explanation is that the high consumption of low-weight organic matter and the difficulty in consumption of larger molecules by the micro-organisms.

Biomass productivity in this experiment was 101.1 mg L^{-1} h^{-1} and specific growth rate was 0.022 h^{-1}, higher than that found by other authors for autotrophic growth and similar to heterotrophic systems [7,12,22].

Fig. 5 presents yield COD to biomass profile suggesting considerable growth with high conversion to biomass in the first 15hours, agreeing with the profiles of biomass and oxygen. These results suggest that it is possible to obtain high yield of this green algae in vinasse as byproduct of sugarcane processing, which is an industrial activity with large-scale operations in Brazil.

Results of *Desmodesmus* sp. counting showed that green algae was growing slowly (Fig. 6). Cell productivity calculated for *Desmodesmus* sp. in this experiment was 13.10^5 cells L^{-1} h^{-1} and points the usual problem of contamination in heterotrophic cultivations, which differed from the conventional total solids

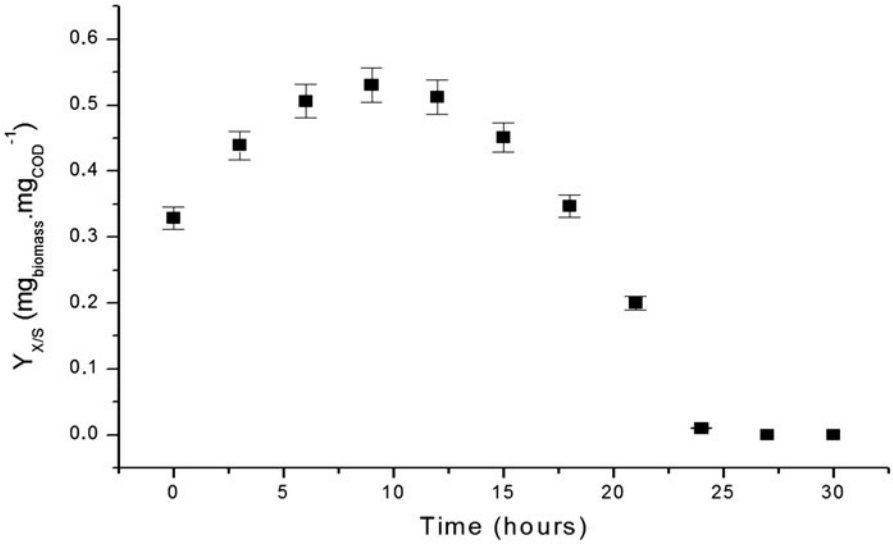

Fig. 5. Yield COD to biomass during the growth of *Desmodesmus* sp. in sugarcane vinasse.

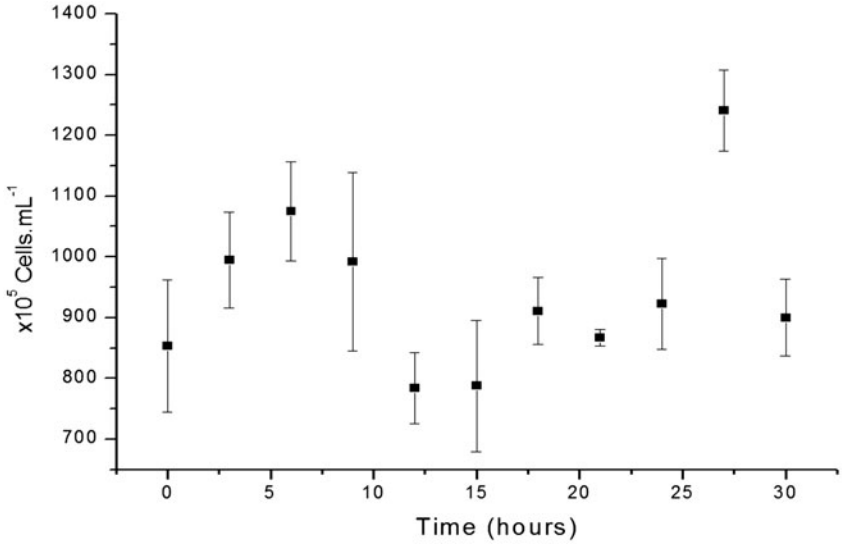

Fig. 6. *Desmodesmus* sp. cells performance in the 30 h experiment.

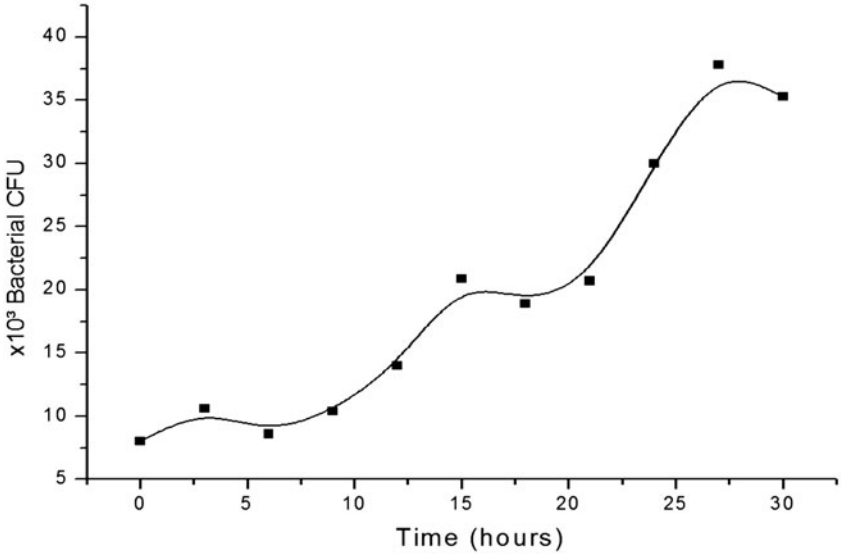

Fig. 7. Bacteria contamination in the 30 h experiment.

analysis that suggest a higher yield COD to biomass (Fig. 5).

However, according to Fig. 7, results of bacteria counting showed that bacteria grew fast in vinasse in an exponential fit, with a lag phase of 9 h. At 10 h-cultivation, the bacterial contamination was still small, which indicated that, at this period, the biomass yield was due to growth of green algae.

Moreover, these results confirm that biomass growth measured by total solids was masked by the bacterial contamination, and that bacteria were responsible for part of the nitrogen and COD consumption. This situation, although very clear when it is heterotrophs crops green algae or cyanobacteria, is not addressed more consistently in the literature. Additionally, these findings also would prevent the application of these micro-organisms since research pointed enhancing in algae systems with bacteria [23].

Bacterial biomass production in this study was of almost 10^3 UFC h^{-1}, which indicates high affinity of bacteria for vinasse's nutrients and a risk of

contamination of eukaryotic microalgal cultivation systems once it put bacteria in a most favorable position for nutrients competition causing stress in green algae that, without light for a mixotrophic metabolism, slows or stops growing by lack of nutrients. Therefore, alternative proposals to minimize problems of bacterial contamination in these heterotrophic cultivations would be to change the composition of the medium without disturbing the algal growth and/or set-up batch of just a few hours.

The literature generally reports the effect of bacteria on the flocculation of microalgae, considering thisan important application because of the difficulties of cell harvesting [24]. Moreover, our results indicate that despite the enhanced bacterial growth after 10 h, it did not cause flocculation and did not change the kinetics of nitrogen and organic matter removal. Thus, the extent of contamination is related more to the quality of microalgal biomass generated.

4. Conclusions

Green algae *Desmodesmus* sp. can be grown in sugarcane vinasse at a specific rate of 0.15 h^{-1} with 36.2 and 52.1% of COD and TN removal, respectively, presenting high and crescent yield of COD to biomass at the first 10 h. Nevertheless, to optimize the growing conditions of green algae in vinasse, the incidence of bacterial contamination, which was monitored in this research, must be controlled. Still, the results shown are promising and help the analysis of the bottlenecks in the production of algae biomass from industrial wastewaters.

Notation

μ_{max} — maximum specific growth rate (h^{-1})
μ — specific growth rate (h^{-1})
CFU — colony forming units(CFU.mL^{-1})
COD — chemical oxygen demand (mg L^{-1})
$E-S$ — percentage efficiency of substrate removal (%)
TN — total nitrogen (mg L^{-1})
P — biomass productivity (mg L^{-1} h^{-1})
TP — total phosphorous (mg L^{-1})
q_{COD} — COD consumption (mg h^{-1})
q_{NT} — TN consumption (mg h^{-1})
R^2 — coefficient of correlation for linear fit
S — final concentration of the substrate (mg L^{-1})
S_0 — initial concentration of the substrate (mg L^{-1})
t — growth time (h)
t_2-t_1 — interval of log phase (h^{-1})
VVM — volume of air per volume of liquid per minute (L L^{-1})
X — final concentration of the biomass (mg L^{-1})
X_0 — initial concentration of biomass (mg L^{-1})
g — relative centrifugal force (g)
$Y_{x/s}$ — biomass yield per substrate consumption (mg mg^{-1})

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