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Chlorella vulgaris growth on anaerobically digested sugarcane vinasse: influenceofturbidity

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Abstract: This paper shows the infl uence of turbidity (in Nephelometric Turbidity Units - NTU), chemical oxygen demand (COD) and aeration (CO_2 supply) on the productivity and growth rate and lipid content of microalgae (a mixed culture predominantly composed of Chlorella vulgaris), using anaerobically digested vinasse as a culture medium. The microalgae can be cultivated in anaerobically digested vinasse, at turbidity and chemical oxygen demand of 690 NTU and 2.5 gCOD L^{-1}, respectively, according to the modifi e d Gompertz model, and removal of turbidity by fi ltration did not infl uence the microalgae productivity (≈ 77 mg L^1 d^1). Furthermore, aeration increased the productivity up to 139 mg L^1 d^1, with a biomass dry weight of 2.7 g L^{-1}. Finally, a maximum lipid content of 265 mg L^{-1} was obtained, while a nitrogen removal of 98% was recorded for all conditions. Thus, the combination of anaerobic digestion followed by the use of the digestate for the cultivation of microalgae may be an effi cient way to treat large quantities of this residue, in turn yielding large amounts of microalgae biomass, which can be transformed into fertilizer and biofuel.

Key words: Biofuel, Gompertz model, lipid, nitrogen, wastewater.

INTRODUCTION

Currently, microalgae cultivation is considered a promising process for obtaining products of great interest, such as biofuels (hydrogen, methane, bioethanol and biodiesel), as well as for producing raw materials for the cosmetic, food and pharmaceutical industries, as well as for the bioremediation of heavy metals, pathogens and organic pollutants in wastewater (Munõz & Guieysse 2006, Christenson & Sims 2011). When compared to conventional terrestrial crops, the mixotrophic and photo-autotrophic cultivation of microalgae has advantages due to their fast growth and the fact that the cultivation neither requires arable land nor competes with food production. In addition, this process does not require large amounts of fresh water, since the microalgae can be grown in saline water or domestic and industrial wastewater like pig manure, distilleries, dairy, fish and cassava processing, among others (Muñoz & Guieysse 2006, Ji et al. 2013, Marques et al. 2013, Posadas et al. 2014).

Essentially, mixotrophic microalgae require light, inorganic and organic carbon, water and nutrients (macro and micro) for growth; the latter can be provided by organic wastes, such as the vinasse produced in ethanol distilleries. However, this wastewater has a low pH (3.5-5.0) and a high chemical oxygen demand (COD) of ≈ 30 g L^{-1} on average (reaching up to 150 g L^{-1}), which can be harmful to the soil and groundwater when applied for fertirrigation

without control (Wilkie et al. 2000, van Haandel 2005, Robles-González et al. 2012, Formagini et al. 2014) This high organic load also requires large amounts of water for dilution or large volume microalgae reactors. Besides, the high turbidity from vinasse can cause a shading effect, decreasing the photosynthetic activity by limiting light availability (Escudero et al. 2014).

In this context, anaerobic digestion can remove much of the organic matter contained in the vinasse, raising the pH during the process (through production of alkalinity, promoting buffering) while maintaining the nutrients in the wastewater basically unchanged, and producing methane, which is useful for energy production (Boncz et al. 2012, Robles-González et al. 2012, Formagini et al. 2014). Hence, the anaerobically digested vinasse (ADV) can be used as an inexpensive source of macro and micro-nutrients for microalgae cultivation and, consequently, for the production of bioenergy as biodiesel. Marques et al. (2013) showed the possibility to integrate microalgae cultivation and the anaerobic digestion of sugarcane vinasse, obtaining a maximum productivity of 70 mg L^{-1} d^{-1} with ≈ 24% of lipids. Despite the promising results, the ADV utilised in these experiments was much more diluted (COD ≈ 0.3 g L^{1} and turbidity ≈ 100 Nephelometric Turbidity Unit - NTU) than the sugarcane vinasse digested in a full scale anaerobic processes (with a COD of 110 g L^{1}) (Siqueira et al. 2013), which requires a more detailed study of the subject in order to improve the microalgae productivity and lipid production.

The present work investigated the effect of turbidity on the microalgae productivity and the production of lipids, using ADV as a culture medium. Furthermore, the influence of COD and aeration (CO_2 supply) was also evaluated to study the improvement of the biomass production on ADV.

MATERIALS AND METHODS

Microalgae consortium

Microalgae were collected from a vinasse storage pond of a sugar and ethanol plant in Mato Grosso do Sul, Brazil, expecting to find species adapted to the vinasse composition. The sample was incubated directly, without the addition of nutrients, under the controlled illumination conditions of a 16:8 hours light:dark photoperiod cycle with light intensity of 47.30 ± 0.67 mmol m^{-2} s^{-1}, from fluorescent lamps, at 23 ± 1ºC. After observing the growth of microalgae, the sample was fixed with formaldehyde, lugol, and 5% acetic acid, according to Sournia (1978), and stored at 4 ºC prior to analysis. The consortium was identified as predominantly Chlorella vulgaris (97%) by microscopic examination (Olympus BX41, USA).

Anaerobically digested vinasse (ADV)

The raw vinasse (20 gCOD L^{-1}) was collected in the same sugar and ethanol plant as explained above and anaerobically digested according to methodology described in Aquino et al. (2007). For this, a 3.0 L glass bottle was filled with 1.93 L of raw vinasse and 0.47 L of anaerobic biomass (0.081gVolatile Solids g_{sludge}^{-1}) collected from a 40 L upflow anaerobic sludge blanket reactor treating vinasse. A substrate/inoculum ratio of 1.0 gCOD g_{Sludge}^{1} and a headspace of 80% were used in the digestion. Sodium bicarbonate ($NaHCO_3$) was used as buffer at a concentration of 0.6 g$NaHCO_3$ $gCOD^{-1}$ (Boncz et al. 2012). The glass botlle was then sealed with a rubber stopper under anaerobic conditions, after oxygen was purged using a gas mixture of 70% N_2 and 30% CO_2, and incubated at 30 ºC. The raw vinasse was digested for about 20 days, until ≈ 85% of COD, relative to non-filtered ADV (ADV-NFT) was removed.

Microalgae batch experiments

The experiments with the microalgae consortium (0.35 ± 0.05 g total suspended solids (TSS) L^{-1}) were performed in 250 mL (Erlenmeyer) photobioreactors (PBRs) with a 100 mL working volume (of which 2% was microalgae culture) and closed with cotton wrapped by gauze, to allow gas exchange. The PBRs were kept in a thermostated incubation chamber at 30 ± 1°C using a 16:8 hours light:dark photoperiod and a light intensity of 47 ± 1 mmol m^{-2} s^{-1}. The temperature was chosen from batch experiments previously performed in order to determine the optimum temperature for the microalgae consortium (data not shown). All experiments were carried out in triplicate and the PBRs were manually shaken and had their position in the incubation chamber changed daily, to avoid some PBRs benefitting from being closer to the illumination source than others.

Part of the ADV was filtered through a 1.2 µm membrane (named of ADV-FT) in order to remove the turbidity. Thus, the influence of ADV-NFT and ADV-FT concentration/dilution, turbidity, and COD on microalgae productivity and lipids production was investigated (Table I). The ADV-NFT and ADV-FT were diluted with distilled water. Finally, ADV-NFT at 98% of ADV concentration was aerated (ADV-AE), with a common 3 W aquarium air *pump to verify the influence of* CO_2 addition (and the better homogenisation). In this case, only this concentration was chosen because in practice it is desireable not to dilute the effluent for cultivation.

The parameters analysed to characterise the initial ADV-FT and ADV-NFT were: pH, turbidity, total suspended solids (TSS), total alkalinity, COD, ammonium ion ($N\text{-}NH_4^+$) and phosphate (PO_4^{3-}) (Table II), according to the Standard Methods (APHA et al. 2005). Total nitrogen was measured using Hach Kits (method 10071). The total carbon (total organic carbon + inorganic carbon) was estimated considering that the COD concentration is about 2.5 times the total organic carbon concentration, as recorded by Martín et al. (2002), while the inorganic carbon concentration was calculated from alkalinity and pH values (Wolf-Gladrow et al. 2007). The parameter used for monitoring microalgae growth in all tests was turbidity (in NTU) (Hanna 93414 turbidity meter), as it provides a direct estimate of biomass concentration (gTSS $\boldsymbol{L}^{-1}$) through the correlation between turbidity and dry biomass content (Toyoda et al. 2011). TSS and soluble nitrogen were also determined at the end of each test. The lipid content was evaluated for ADV-FT and ADV-NFT conditions using the Soxhlet procedure described by APHA

Table I. Conditions of batch experiments performed to assess the effect of initial ADV concentration/dilution, turbidity and COD on the microalgae productivity and lipids production.

ADV-NFT[1]/ADV-AE[2]*			ADV-FT[3]		
ADV Dilution	**Turbidity**	**COD**	**ADV Dilution**	**Turbidity**	**COD**
(%, v/v)	**(NTU)**	**(g L^{-1})**	**(%, v/v)**	**(NTU)**	**(g L^{-1})**
10	34	0.3	20	11	0.6
20	67	0.6	40	14	1.1
50	185	1.6	60	18	1.7
70	267	2.2	80	21	2.3
98	417	3.2	98	24	2.7

[1]NFT – Non-filtered; [2]AE – Aerated; [3]FT – Filtered; *only at 98% of ADV.

Table II. Initial physicochemical characteristics of cultivation media for 98% ADV.

Initial Characteristics	Unit	Cultivation medium	
		98% ADV-FT	98% ADV-NFT/AE
pH	-	7.6	7.6
COD	g L^{-1}	2.7	3.2
Turbidity	NTU	24	417
Total nitrogen	mgN L^{-1}	110	148
Ammonium ion	mgN-NH_4^+ L^{-1}	64	61
Phosphate	mgPO_4^{3-} L^{-1}	191	216
Total alkalinity	g$CaCO_3$ L^{-1}	4.0	3.5
TSS	gTSS L^{-1}	0.03	0.47
C/N ratio	-	27	21

et al. (2005). The experiments were conducted for 35 days. A steady state was reached after ≈ 19, 30 and 16 days for the ADV-FT, ADV-NFT and ADV-AE conditions, respectively, steady state was assumed to occur when the turbidity in the PBRs remained stable for at least three consecutive samplings.

The microalgae productivity (mg L^{-1} d^{-1}) was calculated from the slope of TSS (obtained from turbidity) versus time. The specific growth rate was calculated by the non-linear modified Gompertz model (Zwietering et al. 1990) Gompertz, Richards, Schnute, and Stannard. Finally, the results were evaluated using an analysis of variance (ANOVA) with a Fisher's least significant difference (LSD) test using a 95% confidence level.

RESULTS AND DISCUSSION

Microalgae quantification

The results of turbidity and TSS (g L^{-1}) measurements during the 35 days of monitoring of the different cultivation conditions (triplicates) showed a linear correlation between the two parameters (TSS= 0.00112 × Turbidity; R^2= 0.9889) (Figure 1). Thus, using this empirical relation, the turbidity of the solution in the PBRs can be used to rapidly determine the concentration of algae without using destructive methods like the conventional method for determination of TSS.

Anaerobically digested vinasse as a growing medium

According to the chemical characteristics of the ADV (Table II), the estimated C/N ratios for ADV-NFT, ADV-FT and ADV-AE ranged from 21 to 27, suggesting a lack of nitrogen for all

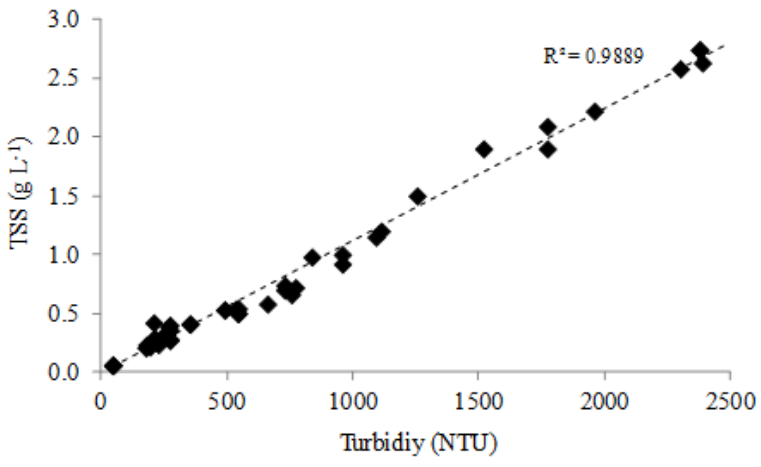

Figure 1. Correlation between TSS and turbidity of microalgal biomass.

conditions, based on the empirical average microalgae biomass formula ($C_{106}H_{181}O_{45}N_{16}P$) (Christenson & Sims 2011). In contrast, a lower C/N ratio of 3.4 was obtained by Serejo et al. (2015) when microalgae from the food industry were cultivated on ADV, with the ADV cultivation medium being limited by carbon. It must be still highlighted that nitrogen limiting conditions can support a much higher lipid content than nitrogen sufficient conditions (Feng et al. 2011).

The biomass productivity increased when the ADV concentration increased in ADV-NFT (R^2= 0.921) and ADV-FT (R^2= 0.8953) (Figure 2). However, no significant differences were found in biomass productivities between ADV-NFT and ADV-FT when the medium contained more than ≈ 45% ADV. In this context, similar productivities were found using 98% ADV-NFT and 98% ADV-FT (76 ± 1 and 77 ± 3 mg L^{1} d^{-1}, respectively). Hence, the microalgal consortium can be cultivated without ADV dilution (98% of ADV and 2% of microalgae, in this case); also, the removal of turbidity was not necessary, since that did not influence the microalgae productivity.

The microalgal biomass productivity increased when the ADV initial turbidity increased in all conditions (Figure 3a). Turbidity up to 417 NTU did not damage the microalgae productivity. According to the modified Gompertz model, a maximum of 85 mg L^{1} d^{1} can be obtained for ADV-NFT, which corresponds to a turbidity of 690 NTU (Figure 3b). Similarly, the biomass productivity increased when the initial COD increased (Figure 3c), with no significant difference obtained when the COD used ranged from 1.8 to 3.2 gCOD L^{-1}. In this context, the modified Gompertz model revealed an optimum of 73 mg L^{1} d^{1} (corresponding to 2.5 gCOD L^{-1}). A comparable productivity (70 mg L^{1} d^{1}) was found by Marques et al. (2013) when cultivating Chlorella vulgaris in batch experiments on ADV (previously diluted with domestic wastewater). However, the authors used a light intensity about 3.5 times higher than the applied in the present work, which significantly interferes with the productivity (Gonçalves et al. 2014) and the cultivation was carried out using a much more digested ADV (≈ 0.2 gCOD L^{1}), which is not feasible in full-scale applications. On the other hand, Ramirez et al. (2014) showed the possibility of using vinasse as a nutrient source for microalgae cultivation at concentrations of up to ≈ 3.0 g L^{-1}

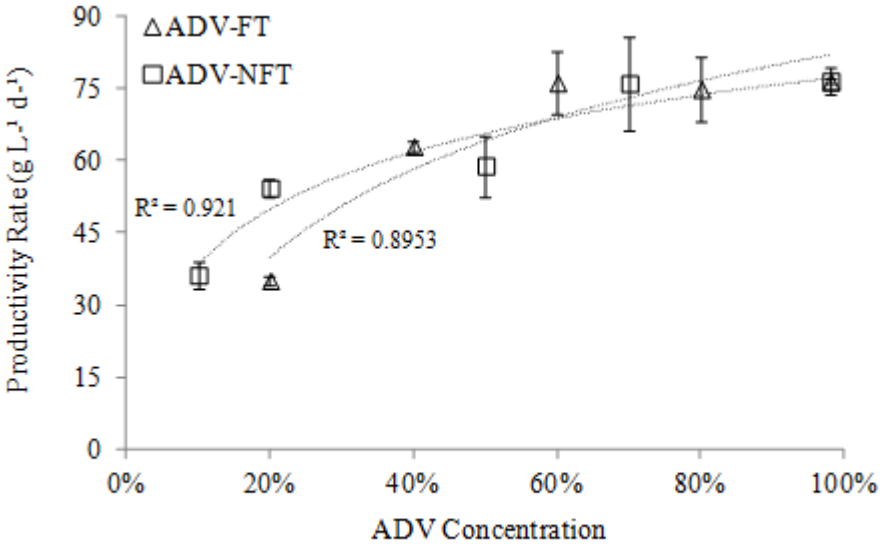

Figure 2. Microalgae productivity in ADV-NFT and ADV-FT at different ADV concentrations/dilutions.

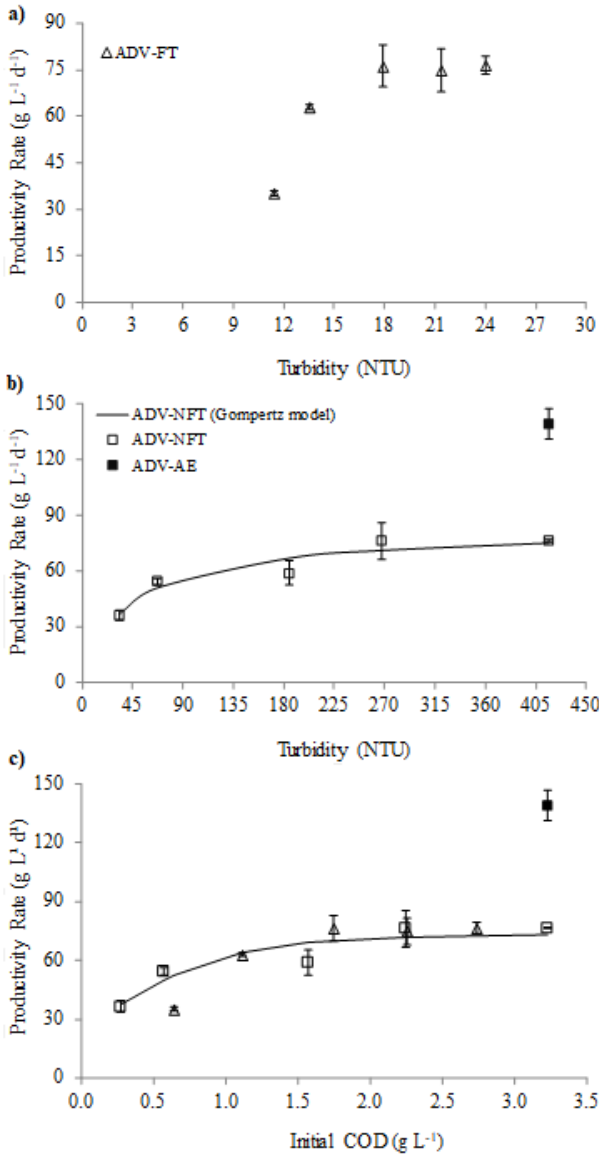

Figure 3. Influence of initial turbidity on microalgae productivity at ADV-NFT (a) and ADV-FT and 98% ADV-AE (b), and influence of initial COD at ADV-NFT and ADV-FT and 98% ADV-AE (c).

of biochemical oxygen demand. However, the vinasse culture medium was supplemented with Guillard Modified Medium.

The better homogenisation by aeration contributed by increasing the productivity from 76 ± 3 (98% ADV-NFT) to 139 ± 8 mg L^{-1} d^{-1} (98% ADV-AE). Barrocal et al. (2010) obtained comparable productivity of ≈ 150 mg L^{1} d^{1} when cultivating *Spirulina maxima* in Schlösser medium supplemented with 5 g L^{-1} beet vinasse (≈ 2.2 gCOD L^{-1}), at a light intensity of 81 mmol m^{-2} s^{-1}, obtaining up to 4.8 g L^{-1} *of biomass. However, the experiments without* vinasse supplementation (only synthetic medium) also recorded high productivity (≈ 0.20 g L^{1} d^{1}) and final biomass concentration (≈ 4.0 g L^{-1}). It must be stressed that the CO_2 did not contribute to the productivity because the ADV is non-carbon limited (Serejo et al. 2015).

A positive correlation was obtained between the microalgae lipids content and initial COD for the ADV-NFT (R^2=0.9893) and ADV-FT (R^2=0.9812) (Figure 4). Furthermore, amounts of 265 ± 4 and 120 ± 5 mg L^{-1} of lipids can be obtained for 98% ADV-NFT and ADV-FT, respectively, corresponding to ≈ 10 and 8% of total content based on dry weight. A higher lipid content (≈ 24%) was obtained by Marques et al. (2013), which corresponded to a maximum amount of only 75 mg L^{-1}, about 3.5 times less than that obtained here. On the other hand, a comparable lipids amount (≈ 290 mg L^{1}) was obtained by Ji et al. (2013) cultivating a similar microalgae (Chlorella vulgaris) on synthetic medium; however, the lipid content decreased directly in response to the amount of pre-treated piggery wastewater supplemented, presenting a lower content of 0.07 g L^{1}.

The lipid content obtained here was unfortunately much lower than those recorded by Feng et al. (2011), of 20-55%, when *Chlorella zofingiensis* was cultivated under nitrogen-limiting conditions. Therefore, further research is necessary to improve the lipid content or even explore other alternatives as carbohydrates for bioethanol production. In this context, Serejo et al. (2015) reported a similar lipid content (≈ 9%), but very high carbohydrate content (≈ 68%) for Chlorella vulgaris cultivated on diluted ADV from the food industry.

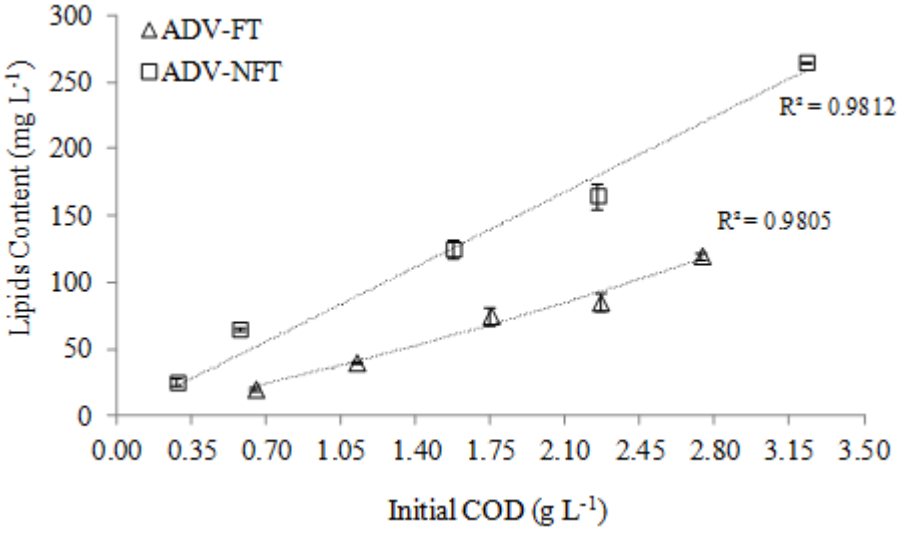

Figure 4. Lipids content in ADV-NFT and ADV-FT at different initial CODs.

The specific growth rates obtained by modified Gompertz model (Table III), for 98% ADV-NFT, 98% ADV-FT and 98% ADV-AE were 0.60 ± 0.02, 0.88 ± 0.06 and 0.86 ± 0.09 d^{-1}, respectively, which were much higher than those obtained by Barrocal et al. (2010) (0.08-0.17 d^{1}), but in accordance with those reported by Marques et al. (2013) of 0.45-0.76 d^{-1}. The lower specific growth rate obtained for 98% ADV-NFT showed a significant influence of turbidity on the consortium cultivation (when compared with 98% ADV-FT), in contrast to the microalgae productivity. On the other hand, the better homogenisation (98% ADV-AE) compensated for the negative effect of turbidity, presenting a higher specific growth rate than 98% ADV-NFT.

The final dry weight of microalgae biomass obtained using 98% ADV-NFT was 2.7 ± 0.1 g L^{1} while the amount of biomass obtained in 98% ADV-FT decreased by 50% to 1.3 ± 0.3 g L^{-1} (Table III). It should be noted though that part of the nitrogen was retained in the filter and consequently the amount of soluble nitrogen available for the mixotrophic microalgae after hydrolysis and sobubilization was also reduced, by about 26%. In addition, the pH increase, which was higher in ADV-FT (> 10), could result in ammonia stripping, reducing available dissolved nitrogen and affecting the amount of biomass produced. On the other hand, despite the microalgae productivity doubling in 98% ADV-AE when compared to 98% ADV-NFT, the amount of microalgae biomass produced in 98% ADV-AE (2.6 ± 0.1 g L^{-1}) was similar ($p<0.05$) to the amount produced in 98% ADV-NFT (2.7 ± 0.1 g L^{-1}), showing that aeration improved the rate of microalgae production, but not the amount (due the nitrogen limitation). In this context, one alternative to increase the amount of microalgal biomass might be adding urea ($CO(NH_2)_2$) to the cultivation media or, as buffer, during the anaerobic digestion of vinasse, as suggested by Formagini et al. (2014).

At this point, it must be highlighted that the final concentration of soluble nitrogen for 98% ADV-NFT, 98% ADV-FT and 98% ADV-AE was less than 3 mgN L^{-1} for all cases, performing a nitrogen removal efficiency of ≈ 98%, which was higher than the maximum obtained by Barrocal et al. (2010) for TKN removal (40-70%). Unfortunately, the elemental biomass N composition was not analysed in order to

Table III. Final physicochemical characteristics of biomass and cultivation media for 98% ADV.

Final Characteristics	Unit	Cultivation medium		
		98% ADV-FT	98% ADV-NFT	98% ADV-AE
Lipid content	mg L^{-1}	120 ± 4[a]	265 ± 2	not analysed
Lipid content/dry weigth	%	9.2 ± 0.1	9.8 ± 0.1	-
Microalgae productivity	mgTSS L^{-1} d^{-1}	76 ± 3[a]	79 ± 1[a]	139 ± 8
Specific growth rate	d^{-1}	0.88 ± 0.06[a]	0.60 ± 0.02	0.86 ± 0.09[a]
Biomass dry weight	gTSS L^{-1}	1.3 ± 0.3[a]	2.7 ± 0.1	2.6 ± 0.1
Soluble nitrogen	mgN L^{-1}	2.4 ± 0.8[a]	3.2 ± 0.7[a]	3.3 ± 0.5[a]

[a] **The values in the same line are statistically equal, ANOVA P-value < 0.05.**

verify the mechanisms of nitrogen removal (into biomass or ammonia stripping). However, considering the obtained nitrogen removal and the N/P ratio of 16 (Christenson & Sims 2011), it can be estimated that the phosphorus removal efficiency was only 12 ± 1%. This removal is similar to that obtained in the treatment of piggery wastewater by Chlorella sp. (de Godos et al. 2010), but inconsistent with the maximum permissible discharge concentration (2 mg L^{-1}) into the environment in European legislation (Posadas et al. 2014), which represents a niche for further research.

In summary, the use of ADV for microalgae cultivation would be most attractive, practically and economically, without any dilution or filtration to remove the turbidity. Therefore, the results show that ADV can indeed be used as a pure culture medium for microalgae production. The estimated annual ethanol production in Brazil was about 26.6 billion litres for the 2013/2014 harvest (Renó et al. 2014). Knowing that the production of every litre of ethanol results in the coproduction of 20 litres of vinasse (Wilkie et al. 2000), and using an output of 2.7 $g_{microalgae}$ L^{1}, Brazil could produce about 1.4 Mt of microalgal biomass (≈ 140 kt of lipids) per year using this waste.

CONCLUSIONS

A microalgae consortium (97% of Chlorella vulgaris) shows similar productivity of 77 and 76 mg L^{1} d^{1} for undiluted ADV-non-filtered and ADV-filtered, respectively, which suggests that the ADV turbidity removal is not necessary. Furthermore, the better homogenisation in undiluted ADV-aerated improved the microalgal productivity to about 139 mg L^{1} d^{1}. According to the modified Gompertz model, the consortium can be cultivated with ADV turbidity up to 690 NTU, with a concentration of up to 3.2 gCOD L^{-1} (optimum ≈ 2.5 gCOD L^{-1}). The lipid content for 98% ADV-NFT was approximately 10% (265 ± 4 mg L^{1}), while ≈ 2.7 g L^{-1} of biomass dry weight was observed. On the other hand, the specific growth rate was influenced by turbidity, with rates of ≈ 0.60 and 0.88 d^{-1} obtained for undiluted ADV-NFT and ADV-FT, respectively. A nitrogen removal efficiency of ≈ 98% was

recorded, while only ≈ 12% of phosphorus was removed. Despite of not reaching a high lipid content, the novelty this work brings resides in the possibility of using ADV as a medium for microalgae growth without the need of dilution or filtration as a pretreatment, which can be an advantage for wastewater treatment combined with energy recovery from microalgae.

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MLS, GR, GBB, PLP and MAB conducted the research. The manuscript was written by MLS and GR. Sampling campaign and quantitative analysis were permormed by MLS, GR and GBB. Data analysis was conducted by MLS and MAB. Review and editing were performed by PLP and MAB. All authors approved the submission of this work.