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Microalgae cultivation in sugarcane vinasse: Selection, growth and biochemical characterization

Hugo Santana [a,b], Carolina R. Cereijo [a,c], Valérya C. Teles [a,c], Rodrigo C. Nascimento [a,c], Maiara S. Fernandes [a,c], Patrícia Brunale [a], Raquel C. Campanha [a], Itânia P. Soares [a], Flávia C.P. Silva [a], Priscila S. Sabaini [a], Félix G. Siqueira [a,b,c], Bruno S.A.F. Brasil [a,c,*]

[a] Embrapa Agroenergy, Brasília/DF, Brazil
[b] Universidade Federal da Bahia, Vitória da Conquista/BA, Brazil
[c] Universidade Federal do Tocantins, Gurupi/TO, Brazil

HIGHLIGHTS

- A biorefinery strategy for microalgae cultivation using sugarcane vinasse is proposed.
- Screening of 40 microalgae allowed the selection of highly productive strains.
- The feedstock obtained can be used to provide proteins, carbohydrates and energy.
- The culture supernatant can be recycled for sugarcane crops fertilization.

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ABSTRACT

Sugarcane ethanol is produced at large scale generating wastes that could be used for microalgae biomass production in a biorefinery strategy. In this study, forty microalgae strains were screened for growth in sugarcane vinasse at different concentrations. Two microalgae strains, *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40, presented vigorous growth in a light-dependent manner even in undiluted vinasse under non-axenic conditions. Microalgae strains presented higher biomass productivity in vinasse-based media compared to standard Bold's Basal Medium in cultures performed using 15 L airlift flat plate photobioreactors. Chemical composition analyses showed that proteins and carbohydrates comprise the major fractions of algal biomass. Glucose was the main monosaccharide detected, ranging from 46% to 76% of the total carbohydrates content according to the strain and culture media used. This research highlights the potential of using residues derived from ethanol plants to cultivate microalgae for the production of energy and bioproducts.

1. Introduction

Microalgae-derived biomass is recognized as an alternative source for an wide variety of bioproducts, such as biofuels, especial oils, pigments and polymers (Perez-Garcia et al., 2011). These photosynthetic microrganisms present higher growth rates and lower land area requirements compared to terrestrial crops commonly used for biofuels production. However, the production of microalgal biomass is still not economically viable due to high costs of cultivation, harvesting and processing (Quinn and Davis, 2015).

Significant cost reductions can be achieved if CO_2, nutrients and water for microalgae cultivation are obtained at low cost (Brasil et al., 2016). Accordingly, the use of several types of waste streams derived from industrial processes, as well as rural/domestic wastewater, have been proposed for microalgae cultivation as a strategy for cost-reduction in microalgae cultivation (Kang et al., 2015).

A potential wastewater for microalgae cultivation is sugarcane vinasse. It is an acid, dark brown liquid, rich in organic compounds (e.g. glycerol, lactic acid, sugars), nitrogen, phosphorus and ions (e.g. K^+, Ca^{2+}, Mg^{2+}) (Ortegón et al., 2016; Parnaudeau et al., 2008). It comprises the main by-product of sugarcane ethanol plants, being generated at 12–14 L per liter of ethanol produced. Although this wastewater is commonly applied in the fertirrigation

* Corresponding author at: Av. W3 Norte (final), Asa Norte, Brasília, DF 70770-901, Brazil.
E-mail address: bruno.brasil@embrapa.br (B.S.A.F. Brasil).

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of sugarcane crops (Dias et al., 2015), its continuous application leads to changes in soil composition and reduces crop productivity (Christofoletti et al., 2013). The use of vinasse for microalgal biomass production has been proposed previously, however these studies showed that the use of sugarcane vinasse, even at low concentrations, can inhibit the growth of microalgae strains (dos Santos et al., 2016; Ramirez et al., 2014).

It is well known that not all strains are able to grow in adverse conditions such as that found in waste streams (Barrocal et al., 2010; Brasil et al., 2016). Therefore, the objectives of this study were to select highly productive microalgae strains capable of growing in sugarcane vinasse and to characterize algal growth requirements and nutrient uptake during cultivation. Additionally, the composition of algal biomass produced in 15 L capacity flat-plate photobioreactors supplemented with CO_2 was analyzed in order to evaluate the strains potential for the production of energy and bioproducts.

2. Materials and methods

2.1. Microalgae strains and inoculum preparation

Axenic microalgae cultures of Embrapa|LBA1 to Embrapa|LBA40 strains (S1 Table) derived from the Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries at Embrapa (Brasília/DF – Brazil) were used. Strains were kept in liquid cultures of Bold's Basal Medium – BBM medium (Nichols and Bold, 1965) containing ampicillin (100 μg/mL), chloramphenicol (25 μg/mL) and amphotericin B (2,5 μg/ml), at 26 °C ± 1 °C, light intensity of 50 $\mu Em^{-2}\,s^{-1}$ and 12/12 h light/dark regimen.

For inoculum preparation, microalgae strains were axenic cultured in BBM using Erlenmeyer flasks under 12/12 h light/dark regimen (light intensity of 100 $\mu\,Em^{-2}\,s^{-1}$), at 26 °C ± 1 °C and aeration with 5 $L{\cdot}h^{-1}$ of atmospheric air. During log phase of growth, these starter cultures were used to inoculate experimental units (i.e.; Erlenmeyer flasks or flat-plate photobioreactors) at an initial absorbance of 0.01 at 680 nm. Inoculum volumes ⩽5% of the working load were used.

2.2. Sugarcane vinasse and medium preparation

Crude sugarcane vinasse was obtained from Jalles Machado (Goianésia/Brazil) ethanol plant. Crude vinasse samples, referred hereafter only as "crude vinasse", were centrifuged at 4800 RCF during 10 min to remove suspended solids and debris. Diluted vinasse formulations, referred hereafter only as "diluted vinasse", were prepared by addition of distilled water to crude vinasse at the proportions indicated (i.e.: 25%, 50% or 75%). Clarified vinasse formulation, referred hereafter only as "clarified vinasse", was prepared as follows: Hydrated lime ($Ca(OH)_2$) was added to crude vinasse (3 $g{\cdot}L^{-1}$). The solution was maintained at rest for 40 min, then centrifuged at 4800 RCF during 10 min and the supernatant was collected. All vinasse-based media formulations were sterilized by autoclaving at 121 °C for 15 min and stored at 4 °C until use. Transmittance of vinasse-based media formulations was measured by spectrophotometry using Spectramax M3 plate analyzer.

2.3. Selection of microalgae for growth in vinasse

Embrapa|LBA1 to Embrapa|LBA40 microalgae strains (N = 40) were screened for growth in sugarcane vinasse. Starter cultures of each strain were inoculated in 250 mL of either diluted vinasse formulations at 25%, 50% and 75% concentration in distilled water or in 100% crude vinasse. Culturing was performed in sterile 500 mL Erlenmeyer flasks aerated with 5 $L{\cdot}h^{-1}$ of atmospheric air, at 26 °C ± 1 °C, light intensity of 100 $\mu Em^{-2}\,s^{-1}$ in a 16/8 h light/dark regimen. Microalgae growth was daily monitored through microscopic inspection during 30 days of culturing.

2.4. Microalgae cultivation

Selected microalgae strains were submitted to cultivation in 250 mL of crude vinasse or BBM (control) under different conditions: I) Axenic culturing using crude vinasse at 12/12 h light/dark regimen; II) Axenic culturing using crude vinasse without light (dark condition); III) Non-axenic culturing using crude vinasse (pH adjusted to 8.0) at 12/12 h light/dark regimen; IV) Axenic culturing using BBM at 12/12 h light/dark regimen. Culturing was performed in 500 mL Erlenmeyer flasks aerated with 5 $L{\cdot}h^{-1}$ of atmospheric air, at 26 °C ± 1 °C, light intensity of 100 $\mu Em^{-2}\,s^{-1}$ in a 12/12 h light/dark regimen.

2.5. Biomass dry weight determination

For biomass dry weight determination, 10 mL samples of the algal culture were collected, centrifuged during 10 min at 10700 RCF and the supernatant discarded. The pellet was washed through three cycles of resuspension in distilled water followed by 10 min centrifugation at 10700 RCF. The washed pellet was dried overnight using a dry oven at 105 °C and weighted.

2.6. Determination of organic compounds concentration in vinasse-based media

The concentration of total reducing sugars (glucose + fructose + sucrose), glycerol, lactic acid and acetic acid in vinasse-based media at the beginning (day 0) and the end (day 8) of microalgae cultivation (Section 2.4) was determined. One milliliter (1 mL) culture samples were collected, centrifuged for 10 min. at 10,700 RCF and the supernatant was analyzed through High Performance Liquid Cromatography analysis (Agilent 1260 Infinity Binary LC System) using Biorad Aminex HPX-87H column (H2SO4 0.005 M, 0.6 mL/min, 45 °C).

2.7. Microalgae cultivation in airlift flat-plate photobioreactors

Selected microalgae strains were cultivated in either diluted vinasse (50%), clarified vinasse or BBM (control) under non-axenic conditions using airlift flat-plate photobioreactors at 13 L of working load (Supplementary Fig. 2). Culturing was conducted for 3 days at 12 h/12 h light/dark regimen (light intensity of 400 $\mu Em^{-2}\,s^{-1}$) and a temperature of 37 °C ± 1 °C during light period and 24 ± 1 °C during dark period. Aeration with 64 $L{\cdot}h^{-1}$ of atmospheric air supplemented with 5% CO_2 was provided.

2.8. Analysis of biomass biochemical composition

After cultivation, algal biomass was harvested by centrifugation at 4800 RCF during 10 min. The biomass was washed three times with distilled water followed by 10 min centrifugation (4800 RCF) and freeze-dried prior biochemical analysis. The following analysis were performed: Total ash content (Van Wychen and Laurens, 2013c); total protein by the Kjeldahl method (AOAC, 1990), using the nitrogen-protein conversion factor proposed for microalgae (Lourenço et al., 2004); total carbohydrates (Van Wychen and Laurens, 2013a); total carotenoids (Huang and Cheung, 2011); and fatty acid (Van Wychen and Laurens, 2013b). For the determination of the calorific value, the microalgal biomass was analyzed using the protocol described by ASTM (2013).

2.9. Determination of carbohydrates profile on harvested biomass

For determination of the carbohydrates profile of the harvested biomasses, the samples were hydrolysed following the methodology proposed by Van Wychen and Laurens (2013a). The carbohydrate profile of the obtained samples was determined using the methodology proposed by Basumallick and Rohrer (2012). The analysis was performed using an ion-exchange chromatography, HPAE-PAD (Dionex ICS 500+ Thermo Scientific), using Dionex Carbo Pac™ SA10-4 μm, 4 × 250 mm and pre-column, with a PAD (Pulsed Amperometric Detection) detector. Elution by gradient with mobile phase composed by 1 mM KOH solution was used. The temperature of the column was 45 °C and 10 °C for the sample. The carbohydrate determination was performed by comparison with the retention times of the analytic standards. Analytical curves of the carbohydrates detected in the samples were used for quantification.

2.10. Determination of vinasse-based media composition

The composition of vinasse-based media at the beginning (day 0) and the end (day 3) of microalgae cultivation in airlift flat-plate photobioreactors (Section 2.7) was determined. Samples were collected, centrifuged for 10 min. at 4800 RCF and the supernatant used for analysis. The following standard methods described by the American Public Health Association (APHA) et al. (2012) were used: SM5210B – Biochemical Oxygen Demand (BOD); QAM.IT.FQ.16A – Chemical Oxygen demand (COD); SM 4500-O/D – Total organic carbon; SM 4500-NO2-B – Nitrite (NO_2^-); SM4500-NH3 – Ammoniacal nitrogen(NH_4^+); SM 4500-P E – Phosphate (PO_4^{3-}); SM 3500-K B - Total potassium (K^+). For nitrate (NO_3^-) levels, was used the method NBR 12620 (1992).

2.11. Statistical analysis

All the experiments were conducted in three independent replicates (N = 3). The results are presented as means of the replicates ± error bars. The analysis of variance used Tukey test with 95% confidence. Statistical analyses were performed using the software GraphPad Prism 5.

3. Results and discussion

3.1. Screening of microalgae strains for growth in vinasse

The screening of forty (40) microalgae strains (Supplementary Table 1) resulted in the selection of two strains, Embrapa|LBA32, a not formally described species of *Micractinium* genus isolated from a natural lagoon in the Amazon rainforest (Hadi et al., 2016), and Embrapa|LBA40, a *Chlamydomonas biconvexa* strain isolated from a sugarcane vinasse stabilization pond from an ethanol plant in Brazil (Hadi et al., 2016) (Supplementary Fig. 1). These strains were able to grow in all media formulations tested, including crude vinasse (data not shown). These results seem to be congruent with the lack of reports in the literature presenting strains capable of vigorous growth in vinasse at high concentrations (dos Santos et al., 2016; Kadioğlu and Algur, 1992).

The selected strains were cultivated in aerated Erlenmeyer's flasks containing 100% crude vinasse under different conditions to evaluate the effect of light and microbial contaminants upon microalgae growth (Fig. 1). Both strains presented similar growth in BBM and crude vinasse under axenic conditions using 12 h/12 h light/dark regimens. On the other hand, algal growth was impaired in the absence of light. These results indicate that both *Micractinium* sp. Embrapa|LBA32 (Fig. 1A) and *C. biconvexa* Embrapa|LBA40 (Fig. 1B) are able to directly uptake crude vinasse nutrients, in a light dependent manner, without need of substrate metabolization by other microrganisms. This result is consistent with the findings of Ramirez et al. (2014), which reported that *Scenedesmus* sp. growth in medium supplemented with vinasse increases in a light dependent manner. In non-axenic algal cultures, crude vinasse pH was adjusted to 8.0 before inoculation to reduce contamination by heterotrophic microrganisms (Fig. 1).

3.2. Uptake of organic compounds present in vinasse during algal cultivation

After cultivation in crude vinasse, the concentrations of glucose, fructose, sucrose, glycerol, lactic acid and acetic acid present in *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 cultures supernatants were evaluated (Fig. 2). When cultivation was performed in the absence of light, no statistically significant change in the concentration of the organic compounds evaluated could be observed (Fig. 2A). This finding is congruent with impaired algal growth under this condition (Fig. 1). When light/-dark cycling is applied to axenic cultures, the culture supernatants of both strains presented a significant reduction in glycerol, but not in Total Reducing Sugars (TRS: glucose + fructose + sucrose), lactic acid or acetic acid concentrations (Fig. 2B). On the other hand, when the microalgae strains were cultured under non-axenic conditions, reductions on TRS, lactic acid and glycerol concentrations were detected (Fig. 2C). Furthermore, a reduction in acetic acid concentration was also observed in *C. biconvexa* Embrapa|LBA40 cultures supernatants (Fig. 2C). The uptake of organic compounds observed in non-axenic cultures (Fig. 2C) is probably associated with the presence of heterotrophic contaminants, such as airborne bacteria and yeasts. Taken together, the results shown in Fig. 1 and Fig. 2 indicate that cultivation of both *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 leads to a decrease in glycerol levels in a light-dependent manner during growth in crude vinasse. This is indicative that strains *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 perform photo-heterotrophic metabolism. The main difference between mixotrophy and photo-heterotrophy is that photo-heterotrophic cultivation requires both organic carbon and light at the same time, while mixotrophy uses either one alternatively (Wang et al., 2014). Accordingly, studies have reported that some microalgae strains can metabolize glycerol as a primary carbon source (Ceron Garcia et al., 2006; Ethier et al., 2011). Furthermore, strains from the genera *Micractinium* and *Chlamydomonas* are able to grow in wastewater by metabolizing both organic and inorganic carbon sources (Bouarab et al., 2004; Wang and Park, 2015).

On the other hand, there are studies that suggest that glycerol uptake can be related to algal cell osmoregulation processes in media with high osmolarity (Anderca et al., 2004; Lin et al., 2013), which is the case of the vinasse (Christofoletti et al., 2013). This uptake could potentially reduce glycerol biosynthesis through starch breakdown, favoring microalgal growth (Goyal, 2007). The determination of the pathway involved with glycerol uptake and metabolization on *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 during growth in sugarcane vinasse, though, remains an issue to be pursued in future studies.

3.3. Cultivation of microalgae in vinasse using airlift flat plate photobioreactors

The scaling-up of the cultivation system is an essential step in order to integrate microalgae cultivation to the ethanol industry in a biorefinery strategy. At large-scale, cultivation parameters cannot be easily controlled, which makes it necessary to use strains that are not easily affected by culture variations and are

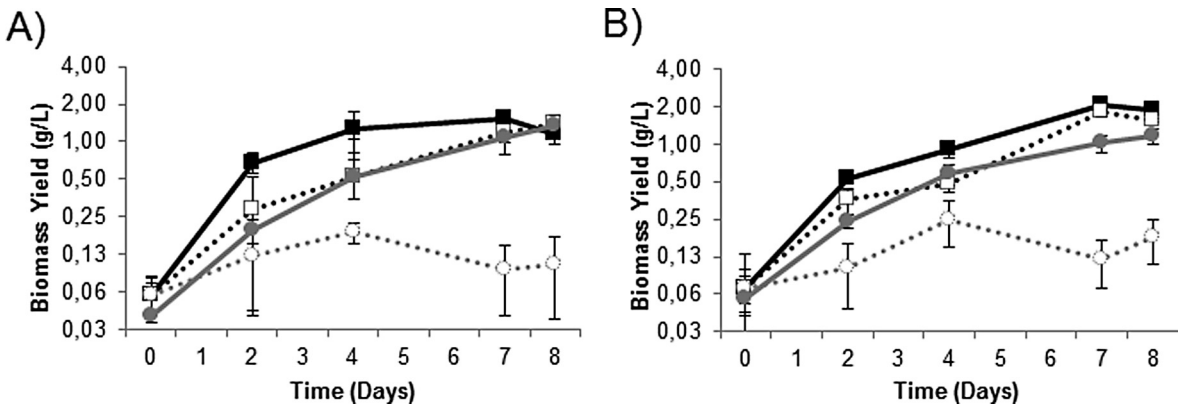

Fig. 1. *Microalgae growth in vinasse.* The strains *Micractinium* sp. Embrapa|LBA32 (A) and *Chlamydomonas biconvexa* Embrapa|LBA40 (B) were grown for eight days using atmospheric air aerated erlenmeyers flasks at 26 °C ± 1 °C under the following conditions: (····○····) Crude vinasse, axenic culture in the dark; (····□····) Crude vinasse, axenic culture, 12 h/12 h light/dark regimen; (—■—) Crude vinasse (pH 8,0), non-axenic culture, 12 h/12 h light/dark regimen and (—●—) BBM, axenic culture, 12 h/12 h light/dark regimen. Experiments were conducted in triplicates (n = 3) and the results are presented as mean ± error bars.

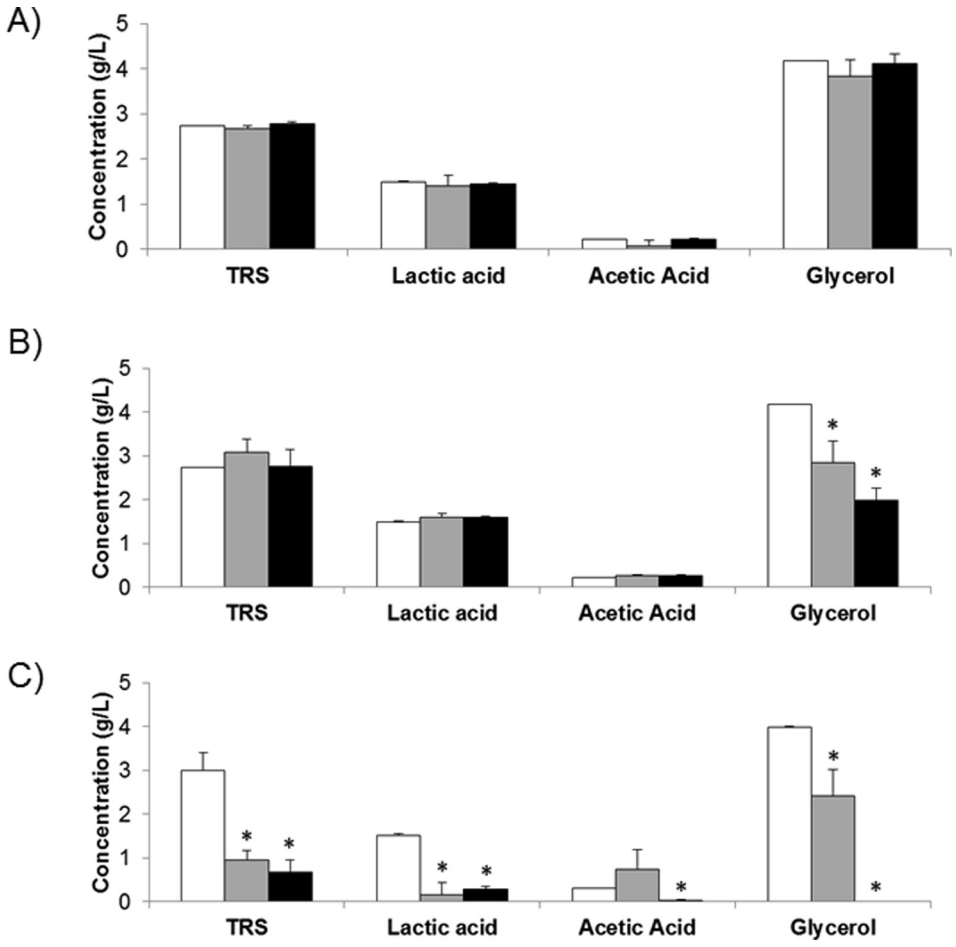

Fig. 2. *Concentration of organic compounds in crude vinasse cultures.* Microalgae strains were grown in crude vinasse for eight days using aerated erlenmeyers flasks at 26 °C ± 1 °C under the following conditions: (A) Axenic under dark; (B) Axenic under 12 h/12 h light/dark regimen; (C) Non-axenic under 12 h/12 h light/dark regimen. The concentration of TRS (Total Reducing Sugars: glucose + frutose + sucrose), lactic acid, acetic acid and glycerol present in culture supernatants was measured through HPLC analysis at day 0 (□) and after 8 days of *Micractinium* sp. Embrapa|LBA32 (■) and *Chlamydomonas biconvexa* Embrapa|LBA40 (■) cultivation. Experiments were conducted in triplicates (n = 3) and the results are presented as mean ± error bars. Paired *t*-test was performed to determinate significant variations in the samples in comparison to uncultured vinasse. Variation was considered significant (*) when p-value is <0.05 compared to the initial condition (day 0).

competitive with contaminants in non-axenic conditions (Christenson and Sims, 2011). This is particularly challenging while dealing with cultivation media with high organic load (i.e.: high BOD and COD), such as vinasse, which promotes the growth of heterotrophic contaminants.

In the present work, the cultivation of Micractinium sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 in vinasse was scaled-up in 15L airlift flat plate photobioreactors aerated with atmospheric air enriched with 5% CO_2 under non-axenic conditions. However, the initial attempts to perform cultivation using

Table 1
Comparison table of biomass productivity obtained with strains cultured under different conditions. The strains *Micractinium* sp. Embrapa|LBA32 (A) and *Chlamydomonas biconvexa* Embrapa|LBA40 cultivated in this study were cultivated in air lift flat plate photobioreactors using BBM (Bold's Basal Medium), 50% diluted vinasse or clarified vinasse. Results are presented as mean ± error bars of triplicate experiments (n = 3).

Strain	Culture conditions	Biomass productivity ($mg{\cdot}L^{-1}{\cdot}d^{-1}$)	Reference
Micractinium sp. Embrapa|LBA32	Bold's Basal Medium	101.11 (±15.75)	This study
Micractinium sp. Embrapa|LBA32	50% water diluted vinasse	177.78 (±5.09)	This study
Micractinium sp. Embrapa|LBA32	100% clarified vinasse	164.44 (±15.75)	This study
C. biconvexa Embrapa|LBA40	Bold's Basal Medium	132.22 (±15.40)	This study
C. biconvexa Embrapa|LBA40	50% water diluted vinasse	182.22 (±16.44)	This study
C. biconvexa Embrapa|LBA40	100% clarified vinasse	222.22 (±10.71)	This study
Spirulina maxima (SAG 49.88)	Schlösser medium supplemented with beet vinasse ($5\ g{\cdot}L^{-1}$)	150.00	Barrocal et al. (2010)
Chlorella vulgaris	Anaerobic digested sugarcane vinasse	70.00	Marques et al. (2013)
Scenedesmus sp.	Guillard Medium supplement with sugarcane vinasse (50%)	24.00	Ramirez et al. (2014)
Botryococcus braunii	Chu 13 medium supplemented with sugarcane molasses ($15\ g{\cdot}L^{-1}$)	113.00	Yeesang and Cheirsilp (2014)
Spirulina maxima	AO medium supplemented with sugarcane vinasse (1%)	47.73	Dos Santos et al. (2016)

crude vinasse (pH 8.0) failed due to outgrowth of contaminants, especially filamentous fungi (data not shown). In addition, since the selected strains grow in a light-dependent manner, the increase in vinasse light transmittance is also required to achieve high algal biomass productivities. In order to circumvent these limitations, three strategies were employed to favor algal growth: (i) elevating pH of vinasse-based media to 8.0 to minimize growth of fungal contaminants; (ii) shortening batch cultures period in photobioreactors to 3 days (end of microalgae exponential growth phase); (iii) increasing light transmittance in the medium through vinasse dilution in distilled water or chemical clarification. The vinasse dilution and clarification processes improved the light transmittance at wavelengths >600 nm up to 74% and 67.5%, respectively, improving the light penetration at the chlorophyll absorption peaks wavelengths (Supplementary Fig. 3). Under these conditions the biomass productivities achieved by *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 using 50% diluted vinasse and clarified vinasse were even higher than that observed for BBM (Table 1). Also, microscopic monitoring of cultures confirmed microalgae as the most abundant organisms present with limited contamination by other microrganisms (data not shown).

In the literature, there are reports of successful cultivation of microalgae strains using vinasse. However, the use of this effluent at high concentrations seems to inhibit algal growth. Barrocal et al. (2010) reported biomass dry weight productivities ranging from 240 to $0\ mg{\cdot}L^{-1}{\cdot}d^{-1}$ in *Spirulina maxima* cultures using synthetic media supplemented with $1\ g{\cdot}L^{-1}$ to $7\ g{\cdot}L^{-1}$ of beet vinasse, respectively. Similarly, studies of Ramirez et al. (2014) reported cultivation of *Scenedesmus* sp. in synthetic medium supplemented with sugarcane vinasse up to 50%, however, the productivities obtained were lower under this condition (i.e.: $24\ mg{\cdot}L^{-1}{\cdot}d^{-1}$ of biomass dry weight). Using a distinct approach, Marques et al. (2013) reported that anaerobic biodigestion of vinasse before *Chlorella vulgaris* cultivation increased algal biomass productivity up to $70\ mg{\cdot}L^{-1}{\cdot}d^{-1}$. In here, *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 strains were grown in 50% diluted vinasse or 100% clarified vinasse and showed average productivities up to $222\ mg{\cdot}L^{-1}{\cdot}d^{-1}$ of biomass dry weight (Table 1).

These findings highlight the importance of screening and selecting robust microalgae strains capable of maintaining high growth rates in wastewater-based cultivation systems. Indeed, it is unlikely that one species/strain of microalgae will thrive in several substrates given the distinct and complex mixtures found in each type of residue/wastewater (Brasil et al., 2016). Therefore, strains will probably be specific for each substrate as indicated by the higher productivities obtained with indigenous microalgae grown in produced water, for example (Brasil et al., 2016; Wood et al., 2015). Not surprisingly, the strain *C. biconvexa* Embrapa|LBA40 selected in the present study is an autochthonous microalgae isolated from a sugarcane vinasse stabilization pond (Hadi et al., 2016).

3.4. Analysis of algal biomass and culture supernatants composition

Microalgae can produce various bioproducts of commercial interest for the industry. Carbohydrates for production of chemicals, proteins for use as animal feed, and lipids for biodiesel, are some examples of such compounds (Benemann, 2013; Pulz and Gross, 2004). The production of these metabolites, however, depends on the microalgae strain and cultivation conditions.

In this study, biomasses and culture supernatants from flat plate photobioreactors batch cultures of *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 strains were analyzed (Tables 2 and 3). The results showed an increase in protein content of microalgal biomass (Table 2), that was paralleled by the significant reduction in nitrate levels on the supernatant of vinasse-based media (Table 3). Another important finding is that the nutrient load of vinasse remains largely unaltered after algal cultivation (Table 3). The growth of neither microalgae strain significantly altered the BOD, COD, organic carbon, NO_2^-, PO_4^{3-} or K^+ content of vinasse-based media (Table 3), allowing further use of culture supernatants for sugarcane crops fertilization/irrigation and contributing to biorefinery integration.

However, a decrease in carotenoids, carbohydrates and fatty acids content during the cultivation of both strains in vinasse formulations compared to BBM was observed (Table 2). Similar results were obtained by Wang et al. (2013), and Del Campo et al. (2007), showing that there is an inverse correlation between protein accumulation and carbohydrates and/or lipid content, including carotenoids, in algal biomass. Since the nitrate levels are significantly higher in vinasse-based media than in BBM, it is possible to hypothesize that the high availability of this nitrogen source is one of the factors that contributed to the accumulation of proteins instead of other compounds (Table 2). It is important to emphasize though, that the higher biomass productivity achieved in vinasse-based media (Table 1) partly compensate the

Table 2
Productivity, composition and year round estimates of microalgal biomass produced in flat-plate photobioreactors. Micractinium sp. Embrapa|LBA32 and *Chlamydomonas biconvexa* Embrapa|LBA40 strains were cultivated for 3 days in BBM, 50% diluted vinasse and clarified vinasse. Results are presented as mean ± error bars of triplicate experiments (n = 3). One-way ANOVA with Tukey post-test was used to evaluate the differences (p-value <0.05). Different letter (a, b, c) means statistically different. Year round estimates were performed considering 200 $m^3 \cdot d^{-1}$ with 240 working days per year.

	Micractinium sp. Embrapa|LBA#32			*Chlamydomonas biconvexa* Embrapa|LBA#40		
	100% BBM	50% Diluted Vinasse	100% Clarified Vinasse	100% BBM	50% Diluted Vinasse	100% Clarified Vinasse
Carbohydrates content (%)	28.21 (±0.24)a	17.55 (±0.03)b	21.79 (±0.36)c	31.61 (±0.18)a	13.50 (±0.76)b	11.71 (±0.14)c
Carbohydrates productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	28.50 (±4.29)a	31.20 (±1.05)a	35.87 (±3.98)a	31.79 (±4.88)a	24.58 (±2.48)b	26.03 (±1.22)b
Protein content (%)	34.03 (±0.10)a	39.50 (±0.47)b	39.62 (±0.09)b	30.96 (±0.17)a	41.68 (±0.35)b	39.92 (±0.60)c
Protein productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	34.40 (±5.36)a	70.20 (±1.20)b	65.15 (±6.20)b	40.94 (±4.94)a	71.27 (±6.29)b	88.71 (±4.83)b
Carotenoid content ($\mu g \cdot g^{-1}$)	669.85 (±10.86)a	192.08 (±24.66)b	26.34 (±3.15)c	805.26 (±174.46)a	165.61 (±22.58)b	12.97 (±0.01)c
Carotenoid productivity ($\mu g \cdot L^{-1} \cdot day^{-1}$)	19.10 (±2.98)a	5.99 (±0.74)b	0.95 (0.18)c	33.94 (9.74)a	4.05 (±0.54)b	0.33 (±0.02)c
Fatty acid content (%)	3.23 (±0.55)a	2.21 (±0.44)a	2.50 (±1.30)a	2.06 (±0.35)a	1.58 (±0.21)ac	1.26 (±0.07)bc
Fatty acid productivity ($mg \cdot L^{-1} \cdot d^{-1}$)	3.22 (±0.45)a	3.94 (±0.90)a	3.99 (±1.64)a	2.73 (±0.64)a	2.88 (±0.50)a	2.79 (±0.26)a
Calorific value ($cal \cdot g^{-1}$)	5037.81 (±16.25)a	5184.49 (±36.85)b	5260.21 (±44.65)b	5069.56 (±34.53)a	5137.54 (±17.86)bc	5114.23 (±19.35)ac
Ash content (%)	3.21 (±0.30)a	6.00 (±0.15)b	5.83 (±0.36)b	5.44 (±0.28)a	6.09 (±0.54)ac	6.67 (±0.31)bc
Year round estimates						
Bio-ethanol ($L \cdot year^{-1}$) [1]	646.55	707.75	813.58	716.91	557.58	590.35
Biodiesel ($kg \cdot year^{-1}$) [2]	155.00	189.01	191.38	98.75	134.36	138.18

Year round estimates considering 200 $m^3 \cdot d^{-1}$ with 240 working days per year (Cabanelas et al., 2013).
[1] Estimated based on the conversion rate of 0.6 L per kg of total carbohydrates (Cabanelas et al., 2013).
[2] Estimated based on the conversion rate of 1 kg of fatty acid to 1 kg of biodiesel (Cabanelas et al., 2013).

Table 3
Vinasse composition after microalgae cultivation using flat-plate photobioreactors. Analysis of the supernatant of 3 days cultures of the strains *Micractinium* sp. Embrapa|LBA32 and *Chlamydomonas biconvexa* Embrapa|LBA40 after 3 days of cultivation in 50% diluted vinasse or clarified vinasse. Results are presented as mean ± error bars of triplicate experiments (n = 3). One-way ANOVA with Tukey post-test was used to evaluate the differences (p-value <0.05). Different letter (a, b, c) means statistically different. N.D. (Not detected. <5.0 $mg \cdot L^{-1}$).

	50% Diluted Vinasse			100% Clarified Vinasse		
	Initial condition	LBA#32 culture supernatant	LBA#40 culture supernatant	Initial condition	LBA#32 culture supernatant	LBA#40 culture supernatant
Biochemical Oxygen Demand ($g \cdot L^{-1}$)	3.74 (±0.20)a	4.98 (±0.21)a	5.36 (±1.45)a	9.64 (±0.69)a	11.73 (±2.09)a	12.23 (±2.50)a
Chemical Oxygen Demand ($g \cdot L^{-1}$)	8.93 (±1.45)a	12.08 (±0.25)a	13.38 (±2.51)a	22.65 (±2.64)a	26.85 (±0.78)a	27.98 (±0.32)a
Total organic carbon ($g \cdot L^{-1}$)	13.14 (±0.68)a	12.59 (±0.29)a	10.87 (±0.97)a	25.66 (±1.85)a	23.80 (±1.95)a	24.42 (±1.47)a
Nitrate ($mg \cdot L^{-1}$)	21.49 (±1.24)a	11.50 (±0.00)b	13.00 (±1.41)b	39.41 (±0.99)a	21.00 (±1.41)b	27.25 (±1.06)c
Nitrite ($mg \cdot L^{-1}$)	0.10 (±0.01)a	0.07 (±0.01)a	0.09 (±0.01)a	0.15 (±0.02)a	0.27 (±0.06)a	0.27 (±0.04)a
Ammoniacal nitrogen ($mg \cdot L^{-1}$)	N.D.	N.D.	N.D.	9.25 (±2.52)a	5.63 (±0.88)a	N.D.
Phosphate ($mg \cdot L^{-1}$)	11.95 (±0.35)a	11.45 (±2.05)a	21.20 (±4.53)a	18.47 (±3.29)ab	19.05 (±0.64)a	15.15 (±1.20)b
Total potassium ($mg \cdot L^{-1}$)	1954.52 (±311.13)a	1368.00 (±223.45)a	1317.00 (±151.32)a	2362.02 (±265.17)a	2996.00 (±82.02)ab	3092.00 (±53.74)b

decrease in these metabolites contents leading to carbohydrates and fatty acids productivities similar to those obtained in BBM cultures (Table 2). This finding contributes positively to the economic feasibility of using this wastewater for microalgae cultivation and its use as feedstock for biodiesel or ethanol production (Table 2) (Supplementary Table 2).

Carbohydrates comprise the second major fraction present in algal biomass produced in vinasse-based media and could be used for the production of ethanol (Chen et al., 2013) (Table 2). The results of carbohydrate characterization also showed that the strains *Micractinium* sp. Embrapa|LBA32 and *C. biconvexa* Embrapa|LBA40 accumulate mainly glucose-based carbohydrates when cultured in

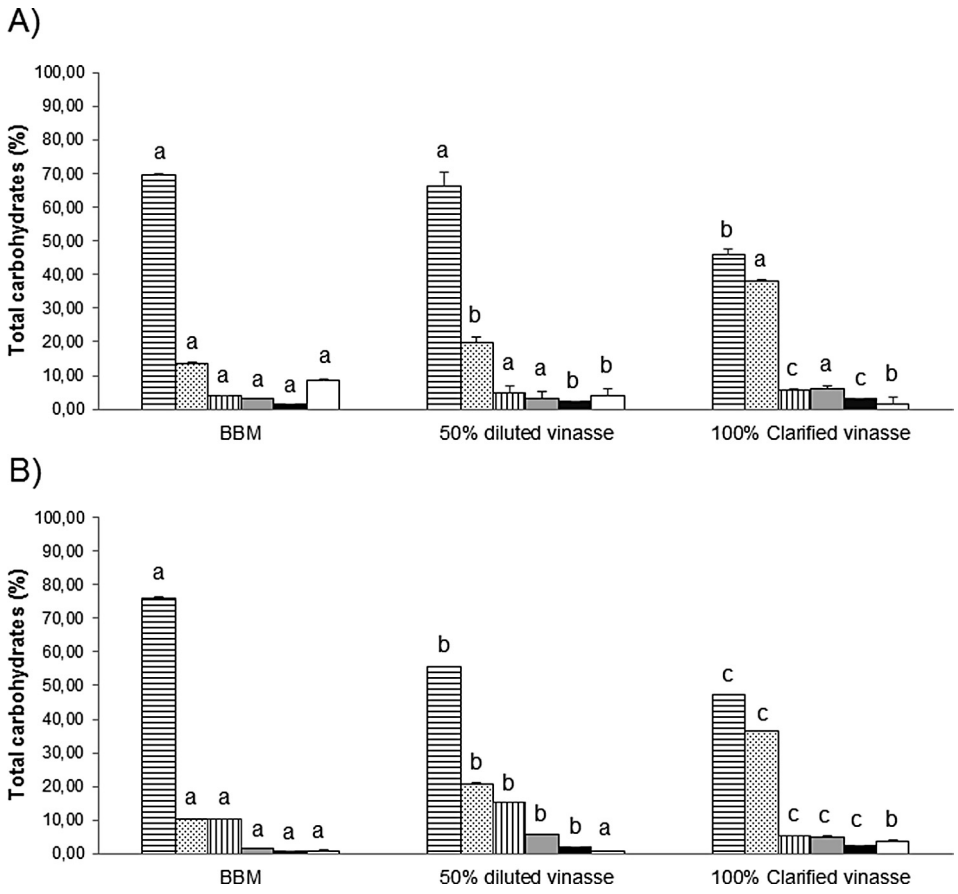

Fig. 3. *Carbohydrate profile of the biomass obtained with the cultivation in air lift flat-plate photobioreactors.* The biomass of the strains *Micractinium* sp. Embrapa|LBA32 (A) and *Chlamydomonas biconvexa* Embrapa|LBA40 (B) cultured in BBM, 50% diluted vinasse and 100% clarified vinasse were analyzed for its carbohydrate profile. Were analyzed the contribution of glucose (▤), galactose (▩), mannose (▥), ribose (▦), myo-inositol (■) and Xylose + Arabinose (□). Experiments were conducted in triplicates (n = 3) and the results are presented as mean ± error bars. Statistical analysis was performed comparing the three media formulations for each carbohydrate separately using One-way ANOVA with Tukey post-test. Differences were considered significant if p-value <0.05. Different letters (a, b, c) means statistically different.

BBM or vinasse (Fig. 3). However, the cultivation in vinasse-based media changed significantly the carbohydrate profile, especially in clarified vinasse formulations, showing the decrease of glucose-based carbohydrates and increase of galactose content for both strains (Fig. 3). Both these sugars are fermentable and can be used for ethanol production, for example, however most fermenting microrganisms cannot metabolize galactose until the depletion of glucose (De Vos and Hugenholtz, 2004). This can limit the application at large scale of vinasse-produced biomass due to reduced overall productivity in fermentative processes (Kim et al., 2012). Factors such as low nitrogen levels, alkaline pH and optimum CO2 supplementation should be used in future studies aiming the optimization of carbohydrate accumulation (Markou et al., 2012).

An alternative application of algal biomass (or its residual fraction) in a sugarcane-ethanol plant would be the generation of power through combustion. In this study, the algal biomasses obtained have calorific values (Table 2) quite similar to that observed for sugarcane bagasse, which is the usual source of biomass for energy cogeneration in ethanol plants (Dias et al., 2011). However, it is important to highlight that the high water content of algal biomasses can represent a major obstruction for its utilization (Jin et al., 2014). Considering that an algal biomass contains up to 60% of water, the specific heat capacity and latent heat of vaporization of water must be considered. In this condition, is possible to estimate that the calorific value of the algal biomass obtained in this study with 60% of moisture is approximately 2054 cal·g^{-1}, in which at least 19% (cal·g^{-1}) of the biomass energy would be necessary to evaporate the biomass water. Furthermore, others factors such as boiler efficiency and steam cycle efficiency, influence the overall efficiency of converting biomass energy in electricity (Luk et al., 2013). Accordingly, the overall energy efficiency obtained from Jin et al. (2014) when using algal biomasses for power generation was around 30%. Therefore, future studies focusing on techno-economic analyzes of the whole process, including the kinetic modelling of microalgae growth, are required to provide critical data on economic and environmental sustainability of this alternative.

4. Conclusions

The isolation and screening of indigenous microalgae strains for the capacity of growing in sugarcane vinasse, allowed the selection of strains with high biomass productivity. The composition of algal biomass produced in vinasse-based media revealed large yields of proteins, carbohydrates and calorific power useful for industrial application. In addition, after algal cultivation, the culture supernatants could be recycled for sugarcane crop fertilization contributing to techno-economic feasibility of the biorefinery. However, there are still issues related to the low light transmittance of vinasse and the control of microbial contaminants in the culture that will pose major challenges to future large-scale cultivation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.12.075.

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