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Growth of Microalgae *Scenedesmus* sp in Ethanol Vinasse

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ABSTRACT

This study evaluated the feasibility of using vinasse as a nutrient source for microalgae cultivation. The Scenedesmus *sp was grown in a medium supplemented with vinasse and process variables were optimized using a factorial design and a Central Composite Design (CCD). The factorial design results showed that it was possible to cultivate microalgae at concentrations of up to 40% of vinasse in the culture medium. The CCD results showed that the light intensity and vinasse concentration influenced the amount of biomass produced.*

Key words: vinasse, microalgae, *Scenedesmus* sp, photobioreactor air-lift

INTRODUCTION

Brazil has been producing ethanol in a large scale for automotive fuel for more than 30 years. The country is the second largest world producer of ethanol and is the only country where the biofuels compete with gasoline (Walter 2009). However, this high production provides a huge amount of waste throughout the years. One inherent byproduct of the process is vinasse. For each liter of ethanol produced, 10 to 18 L of vinasse are generated (Christofoletti et al. 2013). The 2011/2012 harvest yielded approximately 23 million cubic meters of ethanol (Unica 2012), which corresponded to at least 230 million cubic meters of vinasse.

Vinasse is usually used as a fertilizer for agricultural soils due to the presence of nutrients such as nitrogen, phosphorus and potassium. Nevertheless, vinasse presents undesirable characteristics such as an acidic pH, high BOD and unpleasant odors when dumped in open areas. Studies have shown that irrigating crops with vinasse could lead to the contamination of underground water through infiltration, limiting its use (Buchler 1987). Another option is to capture it in a deep well, but underground storage is limited. Brazilian local environmental agencies such as CETESB have established norms for the appropriate discharge of vinasse (CETESB 2006). There are other method such as evaporation to produce animal food and incineration for potassium recovery, but these treatments require high costs (Navarro et al. 2000; Dos Santos et al. 2013). Given the importance of ethanol for the national economy and new restrictions on the use of vinasse, new alternatives should be evaluated. In this context, bioremediation using microalgae could be a promising alternative.

Microalgae have been used at a global scale for different purposes. Aside from their high content of lipid and carbohydrate, microalgae are considered excellent raw material for biofuel production. Microalgae have other advantages as well, such as their fast growth, non-competition with food cultivations and high efficiency in fixing CO_2 (Zhao et al. 2013). Due to their composition, microalgae can be used to obtain pigments and

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also as a nutritional supplement (Brennan and Owende 2010). They can also be used to treat the industrial effluents and sewage. Doria et al. (2012) used *Scenedesmus acutus* inoculated in wastewater from anaerobic digestion process that resulted in a production of 0.74 g/L of biomass in eight days. Oliveira (2013) observed that *Scenedesmus* sp could be used to treat up to 30% of domestic effluents (vinasse has similar characteristics) and obtained a biomass production of 0.48 g/L in ten days.

Many products derived from these microorganisms, such as biofuels, are still expensive due to several reasons, including the nutrients required for the biomass growth. Thus, the aim of this study was to use vinasse as a source of nutrients in order to provide a promising alternative for cultivating microalgae.

Using vinasse pre-treated by an anaerobic process, España-Gamboa et al. (2011) were able to determine the presence of favorable conditions for microalgae growth. Treatment of beet vinasse using a vinasse concentration of 5 g/L (supplemented with Schlösser medium) pre-treated anaerobically using the microalgae *Spirulina maxima* resulted in a production of 4.8 g/L of biomass in eleven days (Barrocal et al. 2010). Marques (2013) worked on diluted vinasse treatment (2 gCOD/L) using *C. vulgaris* and obtained 0.49 g/L in seven days. There is currently no information on specific studies related to the use of *Scenedesmus* sp to treat this effluent.

The aim of this study was to evaluate the viability of producing microalgae *Scenedesmus* sp using vinasse as an alternative culture medium that could replace the Guillard Modified Medium.

MATERIALS AND METHODS

Microorganism

Microalgae cultures were maintained in 1000 mL Erlenmeyer flasks with the Guillard Modified culture medium and adapted at different percentages of vinasse at 25°C and 3910 lux. When the growth reached the exponential phase, medium volume was increased by a factor of two.

Culture medium

Although the Guillard Medium's original composition contained vitamins (Stein 1979), they were not added here. The modified medium contained two different solutions which contained (g/L) macronutrient solution comprising 36.76 $CaCl_2 \cdot 2H_2O$, 36.97 $MgSO_4 \cdot 7H_2O$, 12.6 $NaHCO_3$, 8.71 K_2HPO_4, 85.01 $NaNO_3$, 28.42 $Na_2SiO_3 \cdot 9H_2O$, and micronutrient solution: 4.36 Na_2EDTA, 3.15 $FeCl_3 \cdot 6H_2O$, 0.01 $CuSO_4 \cdot 5H_2O$, 0.022 $ZnSO_4 \cdot 7H_2O$, 0.01 $CoCl_2 \cdot 6H_2O$, 0.18 $MnCl_2 \cdot 4H_2O$, 0.006 $Na_2MoO_4 \cdot 2H_2O$. Different quantities of these solutions were used in the photobioreactors. The vinasse was supplied by COOPERCANA Ltda. (Porto Xavier, Rio Grande do Sul, Brazil), which contained (mg/L) 7410 BOD, 245.51 N, 24.5 P, 89.18 Ca, 557.57 K and 2.30 Na (pH 4.0).

Operation of photobioreactor

Air-lift photobioreactors were used to cultivate microalgae. They were made of acrylic with dimensions of 35 x 17 x 6 cm (Fig. 1), with a central plate of 27 x 17 cm. The culture volume was approximately 3L. Two porous stones placed in the lower riser provided aeration. Heat exchange in the reactor was attained through a stream of water flowing through 316 stainless steel ¼" tubes.

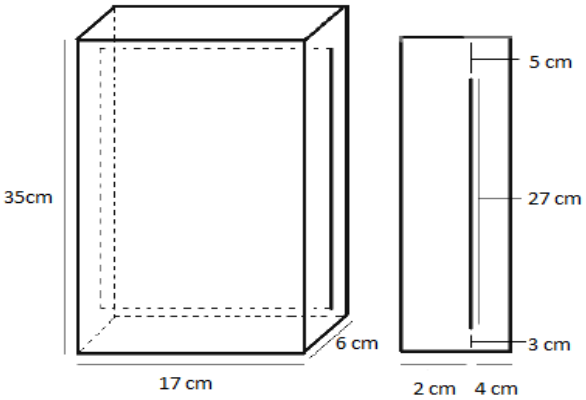


Figure 1 - Air-lift photobioreactor.

The air supply was controlled in the photobioreactors by flow meters. Light intensity was measured on the surface of each photobioreactor. The temperature of the fluid in the refrigeration system was controlled with a digital temperature controller (TIC-Fullgauge 17RGTi). To avoid the loss of culture medium by evaporation, a flask on the photobioreactor lid was added, which allowed the evaporated liquid to condense. A silicone lid covered the orifice sampling to prevent the evaporation (a detailed description of the laboratory apparatus can be obtained in the work of Gris et al. (2013)).

Biomass measurement

Dry weight and optical density (OD) were measured daily to determine the microalgae growth and relationship between the dry weight and optical density. OD was measured at 570 nm wavelength (UV-visible spectrophotometer UV-1600 Pró-Análise) and the dry matter was measured by filtering the samples through 0.7 μm pre-weighed membranes, which were dried at 100°C for 24 h.

Preliminary evaluation of microalgae growth in vinasse

Erlenmeyer flasks (250 mL), in triplicate, containing (%) 0, 12.5, 25, 37.5 and 50 vinasse in modified Guillard medium were incubated on an orbital shaker (model CT-712RN Cientec) at 125 rpm, 27.5°C and light intensity of 6200 lux.

Experimental design

Two experimental designs were developed after the above study. The first was a factorial design involving temperature, light intensity and percentage of vinasse as variables and the second was the Central Composite Design (CCD) with a photoperiod of 12h/12h. Table 1 shows the variables assessed and their levels.

A total of 19 runs were made in CCD (Table 2). The vinasse percentages were altered in this design, with values between 0 and 40%.

Table 1 - Values used in Factorial Design with Vinasse.

Variable	Code	-1	0	1
Temperature [°C]	x_1	20	27.5	35
Vinasse [%]	x_2	0	25	50
Light Intensity [Lux]	x_3	2400	6200	10000

Table 2 - Values used in CCD with Vinasse.

Variable	Code	-1.68	-1	0	1	1.68
Temperature [°C]	x_1	20	23	27.5	32	35
Vinasse [%]	x_2	0	8.1	20	31.9	40
Light Intensity [Lux]	x_3	2400	3940.5	6200	8459.5	10000

RESULTS AND DISCUSSION

Growth curve determination

The relationship between the biomass and OD_{570} is given by:

$$\text{Biomass } [g.L^{-1}] = 0.4669 \cdot OD_{570nm} - 0.0243 \quad R^2=0.9889 \quad (1)$$

This relationship was used as an approximation to dilute the stock culture and reach the desired concentration. The end of the exponential phase was reached after 6 to 10 days of cultivation. Thus, the experiments were maintained for 10 days.

Microalgae growth at different percentages of vinasse

Figure 2 shows the concentration of biomass produced as a function of percentage of vinasse added to Guillard Modified Medium (blended with up to 50% vinasse); when vinasse content increased, the biomass production decreased.

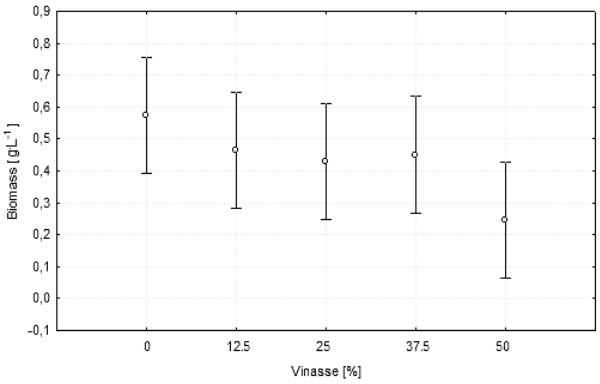


Figure 2 - Growth of Scenedesmus sp at different percentages of vinasse in shaker.

Experimental Design

Factorial Design

Table 3 shows the results of the analysis of variance (ANOVA) at a confidence level of 95%. The bold fonts denoted significant results.

Table 3 - Analysis of variance - factorial design considering temperature, percentage of vinasse and light intensity.

Factor	SS	df	MS	F	p
(1)Temperature [°C]	**0.156157**	**1**	**0.156157**	**76.7626**	**0.012778**
(2)Vinasse [%]	**0.203448**	**1**	**0.203448**	**100.0096**	**0.009852**
(3)Light Intensity [Lux]	**0.196262**	**1**	**0.196262**	**96.4772**	**0.010207**
1 by 2	**0.045346**	**1**	**0.045346**	**22.2908**	**0.042052**
1 by 3	0.018193	1	0.018193	8.9431	0.095989
2 by 3	**0.094692**	**1**	**0.094692**	**46.5483**	**0.020815**
Lack of Fit	0.004965	2	0.002482	1.2202	0.450401
Pure Error	0.004069	2	0.002034		
Total SS	0.723130	10			R^2= 98.8%

Results showed that the percentage of vinasse was the most significant factor, followed by light intensity and then temperature. Evidently not only the linear factors were significant, the interactions between the light intensity-vinasse concentration (2 by 3) and temperature-vinasse concentration (1 by 2) were significant as well. The variance associated with the lack of fit did not show significance for a confidence level of 95%. The interaction between the light intensity-vinasse concentration could be explained by the fact that the concentration of vinasse affected the penetration of light in the culture. The full model, which was adjusted by using all the parameters, presented R^2 equal to 98.8%. The reduced model, adjusted with only significant parameters presented an R^2 equal to 96.2%.

The reduced model for biomass in $g \cdot L^{-1}$ as a function of temperature (x_1), vinasse (x_2) and light intensity (x_3) within the range provided in the Table 1, could be given by:

$$\text{Biomass} = 0.488 + 0.1397\, x_1 - 0.159\, x_2 + 0.157\, x_3 + 0.075\, x_1 x_2 + 0.156\, x_1 x_3 \quad (2)$$

Figure 3 shows the response surface model based on the Equation (2). It showed that light intensity and temperature contributed positively to biomass production. However, when the percent of vinasse was increased in the medium, biomass production decreased. Figure 3A showed that higher values of light intensity produced more biomass when compared to a biomass production at low light intensity without vinasse. At 10000 lux (maximum point of the interval), good biomass production was achieved with up to 40% vinasse. Based on these results, the concentration of vinasse was set between 0 - 40% in CCD studies.

CCD Studies

The inoculums were adapted to different vinasse percentages before using them in the CCD experiments in the air-lift photobioreactors. Cultivation lasted 10 days. The analysis of variance (Table 4) showed that the light intensity and vinasse percentage were the most statistically significant parameters, whereas the temperature had no significant effect within the studied range. The linear terms were associated with the letter L and the quadratic terms with the letter Q.

The full model, which was adjusted by using all the parameters, presented R^2 equal to 90.9%. The following equation gave the reduced model encoded for the determination of biomass in $g \cdot L^{-1}$ as a function of vinasse (x_1) and light intensity (x_3) within the range provided in the Table 2:

$$\text{Biomass} = 0.3912 - 0.0953\, x_1 + 0.0915\, x_3 \quad (3)$$

Figure 4 presents the response surface for the reduced model based in the Equation (3). It showed that with increasing light intensity, biomass production also increased but increase in the concentration of vinasse exerted a negative effect on growth.

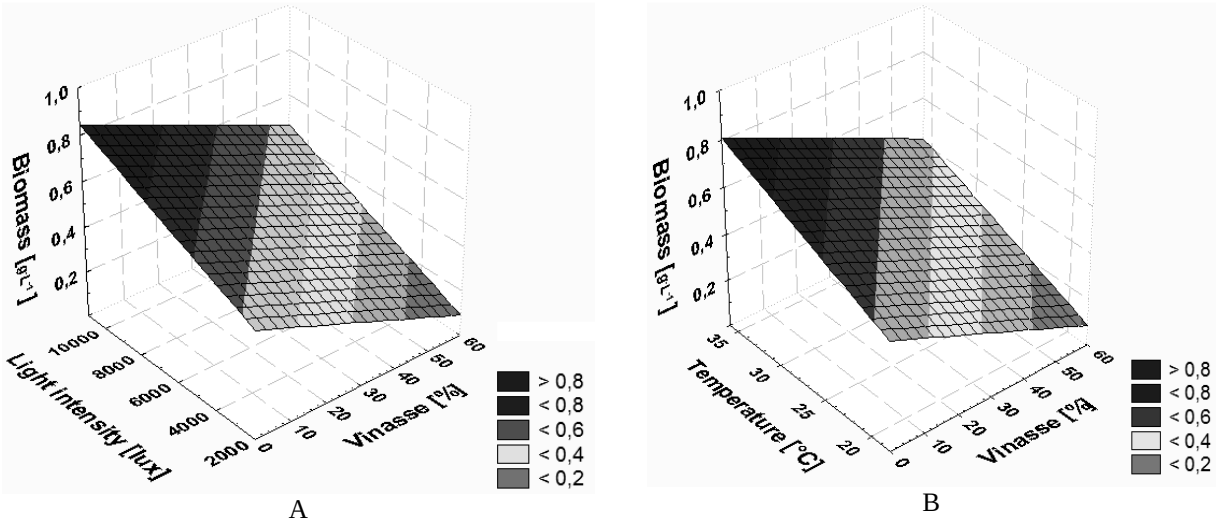
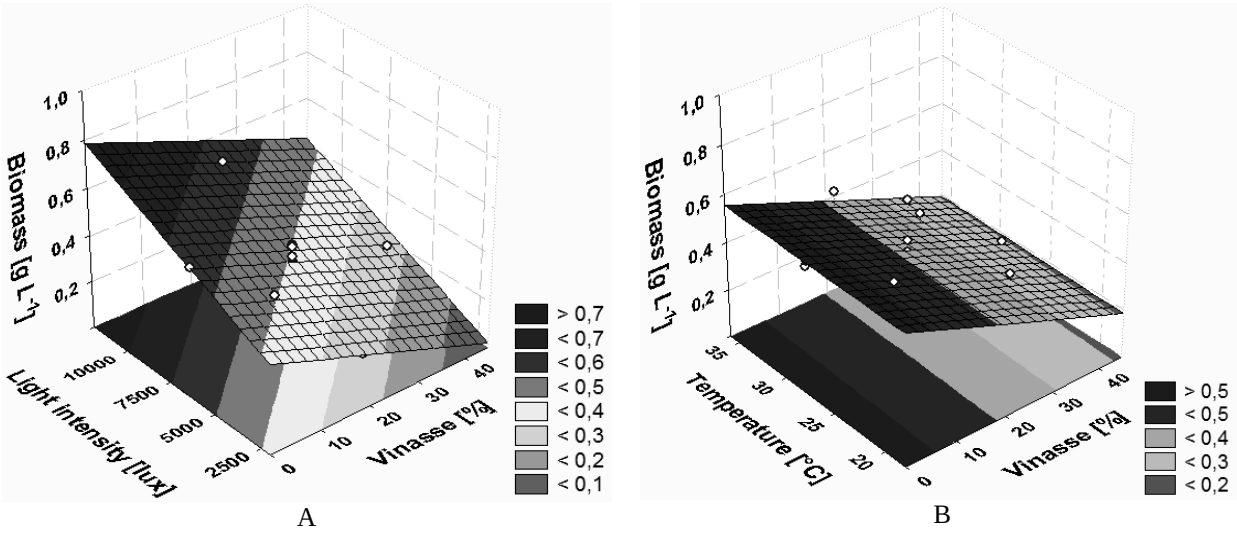


Figure 3 - Response surface of the reduced model for production of the Scenedesmus sp using vinasse - Factorial Design (A) vinasse - light intensity - biomass (B) vinasse - temperature - biomass.

Table 4 - Analysis of variance - Central Composite Design considering temperature, percentage of vinasse and light intensity.

Factor	SS	df	MS	F	p
Blocks	0.013122	1	0.013122	9.06401	0.057182
(1)Vinasse [%](L)	**0.124128**	**1**	**0.124128**	**85.74348**	**0.002665**
Vinasse [%](Q)	0.000595	1	0.000595	0.41095	0.567097
(2) Temperature [°C] (L)	0.000071	1	0.000071	0.04935	0.838468
Temperature [°C] (Q)	0.000720	1	0.000720	0.49741	0.531470
(3)Light Intensity [Lux](L)	**0.114447**	**1**	**0.114447**	**79.05674**	**0.003000**
Light Intensity [Lux](Q)	0.000642	1	0.000642	0.44328	0.553161
1L by 2L	0.006882	1	0.006882	4.75354	0.117320
1L by 3L	0.002790	1	0.002790	1.92721	0.259191
2L by 3L	0.000652	1	0.000652	0.45051	0.550152
Lack of Fit	0.022189	5	0.004438	3.06548	0.192722
Pure Error	0.004343	3	0.001448		
Total SS	0.290351	18			R^2= 90.9%



A B

Figure 4 - Response surface of the reduced model for production of the Scenedesmus sp using vinasse – CCD (A) vinasse - light intensity - biomass (B) vinasse - temperature – biomass.

The design was used to evaluate and statistically select significant factors as well as identify the effects that influenced the biomass production. From these observations, the temperature was excluded. This demonstrated that it was possible to cultivate *Scenedesmus* sp within the temperature range established due to its low significance. Only an increase in vinasse could have a negative impact on the growth. Nonetheless, if increasing concentrations of the effluent were desired, the light intensity should be proportionately higher in order to maintain the same biomass production. The pH in all the photobioreactors increased to about 7.0-8.0, which was a positive factor considering that vinasse pH was 4.0. Doria et al. (2012) and Oliveira (2013) demonstrated the growth of microalgae using wastewater. The results of Oliveira (2013) were similar to those obtained in this study, which obtained a production of 0.48 g/L in cultures with 28% of vinasse. Barrocal et al. (2010) showed that, in batch culture, *Spirulina maxima* was able to grow on Schlösser medium containing up to 5.0 g/L of diluted beet vinasse, reaching biomass concentrations between 3.5 to 4.8 g/L. The vinasse concentration applied in this study was similar to that used in Marques's (2013), where vinasse concentration was 2.0 g/L and biomass concentration was 0.7 g/L in a 10 days batch. In this study, the biomass concentration was

equivalent when the equivalent percentage of vinasse was used. However, vinasse concentration was higher than others available in the literature. Moreover, there was no pre-treatment of the vinasse, as proposed in the literature.

CONCLUSIONS

This study showed that it was possible to grow *Scenedesmus* sp. in vinasse, with percentages up to 40%. The maximum point of the exponential phase was achieved between the 6th and 10th day. The factorial design study showed that the use of 50% vinasse in the culture medium resulted in a smaller amount of microalgae biomass; the Central Composite Design demonstrated that it was possible to cultivate microalgae at concentrations up to 40% vinasse in the culture medium. This design showed that light intensity and percentage of vinasse influenced the amount of biomass to be produced. Additionally, temperatures between 20 and 35°C had no significant effect when working with percentages smaller than 40% vinasse. Thus, the study demonstrated that vinasse could be used as a nutrient source for microalgae production.

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