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Validation, characterization and comparison of microalgae *Chlorella vulgaris and Chlamydomona reinhardtii* growth kinetics

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Abstract : Abstract: Growth bioassays of microalgae Chlorella vulgaris and Chlamydomona reinhardtii were performed at biotechnology laboratory of SENA - Centro Internacional Náutico Fluvial y Portuario, Colombia using modified Conway medium. For microalgae cell count, neubauer chamber method was used and also optical density was measured by spectrophotometry at 685 nm for this purpose. The results were validated using analysis of variance (ANOVA), obtaining 95% confidence in the culture method. linear relationships between the population growth and optical density parameters for both microalgaes were found with an R^2 of 83.4% for Clhorella and 89.3% for Chlamydomona. The characterization and comparison of growth kinetics, showed a significant difference between the growth of the species, reaching maximum growth values of 5'082,500 cel · mL^{-1} for Chlorella and of 1'590,000 cel · mL^{-1} for Chlamydomona, this was verified with the kinetic parameters obtained from the culture of the species.
Key words : microalgae, *Chlorella vulgaris*, *Chlamydomona reinhardtii*, statistics, kinetics.

Introduction

Microalgaes are single-celled photosynthetic organisms with a simple structure that allows their rapid cell growth, because of this they may produce a large quantity of biomass in short periods of time under controlled conditions(Li, Huang, Sandmann, & Chen, 2009). Microalgaes require different factors to grow such as light, being this their main source of energy along with carbon dioxide(Martin, 2010). Others limiting factors for microalgaes growth are: temperature, salinity, pH, photoperiod and the addition of nutrients(Krzemińska, Pawlik-Skowrońska, Trzcińska, & Tys, 2014).

Two microalgaes with biotechnological potential are: The microalga Chlorella vulgaris that belongs to the Chlorophyceae, it has spherical cells, with a chloroplast in the form of a cup and a pyrenoid. The genus Chlorella is cosmopolitan and therefore adapts to different environmental and nutritional conditions(Sheath & Wehr, 2003)Another specie is Chlamydomona reinhardtii which is a flagellates genus microalgae and is part of Chlorophyceae (green algae) in the Chlamydomonadales group(McNeill et al., 2012).

Biotechnology interest in microalgae has been intensified recently due to the particularity of many microalgae to produce different products such as antibiotics, pigments, steroids and other compounds of high added value and commercial interest such as astaxanthin and β-carotene, lutein,(Brennan & Owende, 2010)(Chisti, 2008)(Suali & Sarbatly, 2012)(Mata, Martins, & Caetano, 2010). These microorganisms may be used in bioremediation processes of water polluted with different substrates, which might become an efficient

alternative for the treatment of wastewater(Olguin & Sanchez-Galvan, 2011)(Larsdotter, Söderbäck, & Dalhammar, 2004).

In order to establish a microalgaes production system, a deep knowledge of isolation techniques, purification and maintenance of strains, as well as the knowledge of its morphology, life cycle, biochemistry, etc., is required. This will determine the feasibility of the final biotechnological use. The aim of this research is the validation of microalgaes Chlorella vulgaris and Chlamydomona reinhardtii culture method through the use of statistical tools, as well as the characterization and comparison of growth kinetics of the microalgaes in laboratory conditions.

Experimental

Materials and methods

Microalgaes

Chlorella vulgaris and Clamydomona reinhardtii were obtained from the Culture Collection of Algae at SENA - Centro Internacional Náutico Fluvial y Portuario, located in the city of Cartagena de Indias, Colombia.

Culture systems and methods

Microalgaes were cultured using batch scaling System, starting from a petri dish to a test tube, then to a 250 ml Erlenmeyer flask and finally to a 1000 ml Erlenmeyer flask. Strains were maintained in Conway medium. Culture conditions included a temperature of 24 ± 2 ° C, fluorescent lamps of 39W as source of artificial illumination with irradiation of 5000 lux, photoperiod of 12 hours of light and 12 of darkness, aeration using mechanical blower, with no CO_2 injection. Each microalgae was cultured four times during eleven days, using the same culture methodology and time until death phase was reached.

Reactives

Modified conway medium (Tompkins et al., 1995) was prepared in Sena biotechnology laboratory with this composition: $FeCl_3 \cdot 6H_2O$ (26 g), $MnCl_2 \cdot 4H_2O$ (0,72 g), H_3BO_3 (67,2 g), EDTA (90 g), $Na_2HPO_4 \cdot 12H_2O$ (40 g), $NaNO_3$ (200 g), Na_2SiO_3 (40 g), H_2O (2 L), traces of metal solution (2 mL) and vitamins solution (100 mL). The traces of metal solution was composed of $ZnCl_2$ (2.1 g), $CoCl_2 \cdot 6H_2O$ (2 g), (NH_4) 6 $Mo_7O_{24} \cdot 4H_2O$ (0.9 g), $CuSO_4 \cdot 5H_2O$ (2 g) and distilled water (100 mL), and the vitamin solution was composed of decamyl (210 mg) and distilled water (100 mL).

Cell growth kinetics

Cell growth was analyzed by taking aliquots of the culture every twenty-four hours and determining the number of cells using neubauer chambers(Arredondo & Voltolina, 2007) Also, the optical density of the cultures was also measured by spectrophotometry at 685 nm using a Genesys 20 spectrophotometer(Baldisserotto, Giovanardi, Ferroni, & Pancaldi, 2014) (Frampton et al., 2013). The specific growth rate was determined by the equation:

$$\mu = \frac{\ln\left(\frac{N_2}{N_1}\right)}{t_2 - t_1}$$

Duplication time (Santos-ballardo et al., 2015) was determined using the equation:

$$t_d = \frac{ln2}{\mu}$$

wherein: μ, specific growth rate; N1, initial cell concentration(cel·mL^{-1}); N2, final cell concentration(cel·mL^{-1}), t_d is duplication time and t, time (days).

Statistical analysis

In order to compare the mean of Chlorella vulgaris and Chlamydomona reinhardtii cells in the four experiments, ANOVA technique was used. A linear regression model was found for growth and absorbance Measurements, then the statistical assumptions were tested. An ANOVA was then performed to compare the growth of the two microalgae and to determine if there were differences between them. Statgraphics 16 and Matlab R2015B were used for all calculations. (Montgomery, Runger, & Hubele, 2009) (Otto, 2016)

Results and discussion

Chlorella vulgaris

For Chlorella vulgaris growth, the four cultures exhibited the behavior shown in Fig. 1

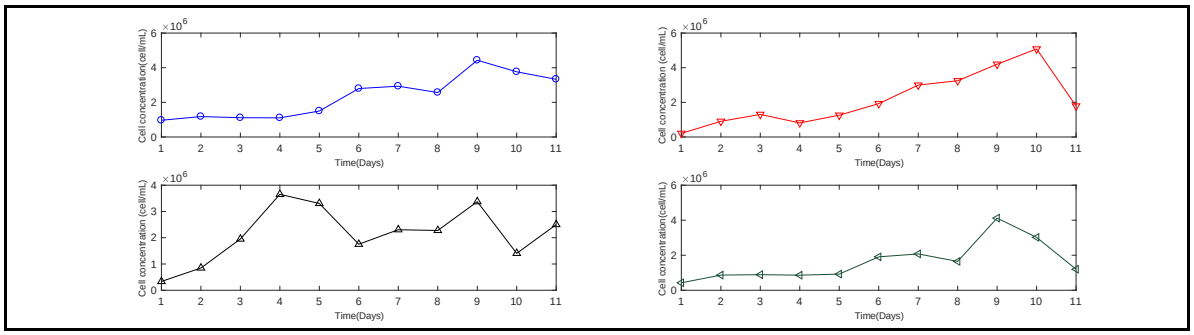

Fig. 1 Chlorella vulgaris growth

Bioassay 3 showed outliers on days 4 and 5, probably due to human error during measurement. In order to verify that an ANOVA study was carried out to compare the behavior of the cultures.

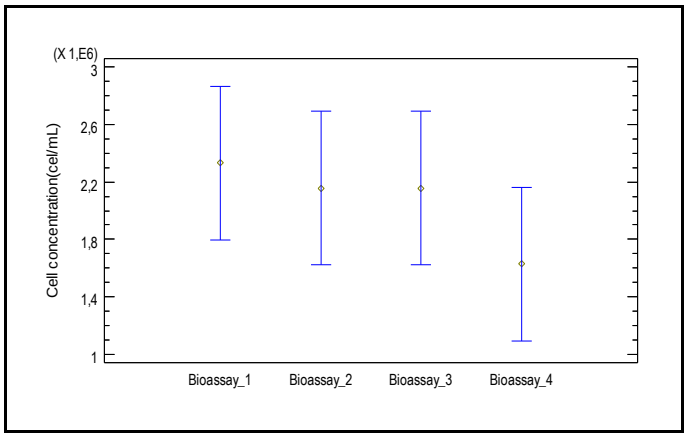

Fig. 2 Growth means comparassion for Chlorella vulgaris

Because of p-value of the F-ratio is greater than 0.05, there is no statistically significant difference between the mean of the four bioassays with a confidence level of 95.0%. (See Fig. 2) With these results it is assumed that the method is appropriate to culture Chlorella vulgaris.

The optical density measurements performed on the four bioassays of Chlorella vulgaris during the culture time are shown in Fig. 3.

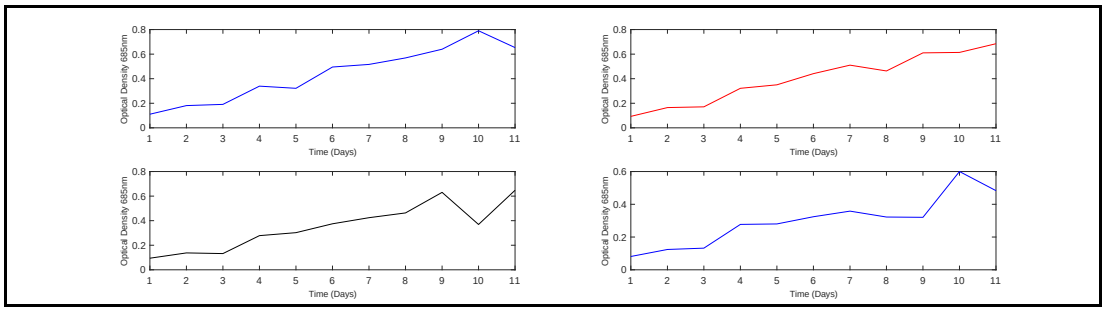

Fig. 3 Optical Density of Chlorella vulgaris

To discard anomalies in the data for optical densities an ANOVA study was performed, it showed a p-value greater than 0.05, this means that there is no statistically significant difference between the mean populations of the four experiments with a 95% confidence level.

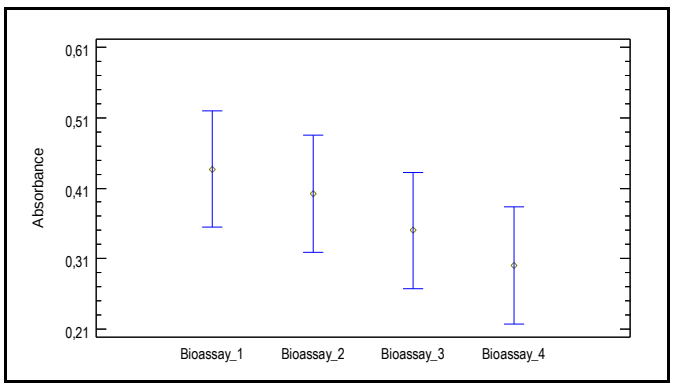

Fig. 4 Comparison of means for optical density of Chlorella vulgaris

Fig. 4 shows that the data obtained in the experiments had a similar behavior, indicating that there is some degree of repeatability in the tests. It may be noticed that experiment four had lower performance than expected, this may be due to problems with aeration of the culture.

In order to find a relationship between population growth and optical density of Chlorella vulgaris cultures, a linear regression analysis was performed. A p-value equals to 0.0001 was obtained, indicating a Relationship between variables. Also for the variables were obtained a Correlation coefficient (R) of 0.92, an R^2 of 85.1%, an R^2 (adjusted) equal to 83.4% and a Durbin-Watson Statistic of 2.59. The correlation coefficient equals to 0.92, indicates a strong relationship between the variables. The R^2 indicates that the adjusted model explains 85.1% of the variability in Population.

The plot that explains the relation between the population and the optical density is shown in fig. 5.

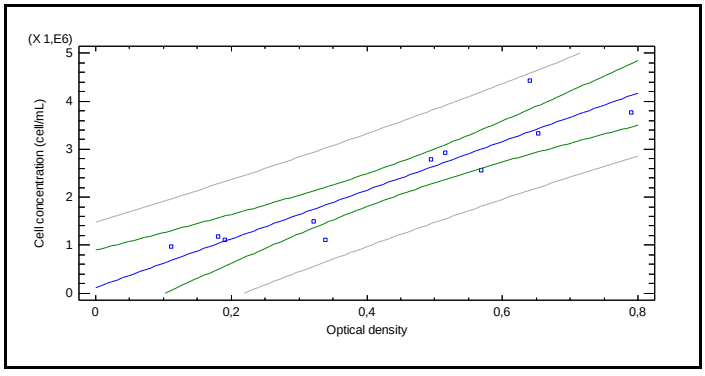

Fig. 5 Relationship between cell concentration and optical density for Chlorella vulgaris

The equation of the adjusted model is:

$$Cell\ concentration(cell \cdot mL^{-1}) = 116335 + 5.0735 \cdot 10^{6} \cdot Optical\ density$$

Shapiro-Wilks test was used to determine the normality of the residues, which yielded a value of 0.94 with a p-value of 0.46. thus the p-value is less than the chosen alpha level, then the null hypothesis is not rejected and there is evidence that the data tested are from a normally distributed population. The Durbin-Watson (DW) statistic examines the residuals to determine if there is any significant correlation based on the order in which they are presented in the data file. Since the p-value is greater than 0.05, there is no indication of a serial self-correlation in the residues. According to this the linear model is accurate to the obtained data.

Chlorella vulgaris growth kinetic

Lag phase covered from day zero (0) to day four (4) where the microalgae was adapting to the established environment conditions. The exponential phase in all the bioassays started between days 4 to 5, in this period the process kinetic is best appreciated. The values for growth rate (μ) and duplication time (td) are shown in Table 1, it shows that the maximum growth rate was reached by bioassays 2 and 4 (0.3) as well as the lowest doubling time (2.3 days). The maximum biomass production observed for Chlorella vulgaris was 5'082,500 cel • mL^{-1} and was reached in the second bioassay.

Table 1. Growth rate (μ) and duplication time (td) for Chlorella vulgaris

μ (1/days)	**T_d(days)**
0,27	2,56
0,30	2,31
0,12	5,77
0,37	1,87

On day 10, the stationary phase began, this is a period in which growth ceases due to the absence of nutrients, the death of the cells is promoted and phase of death or decline begins. It continued until day 11.

Chlamydomona reinhardtii

Data obtained for Chlamydomona reinhardtii growth, using a neubauer chamber for cell counting, is depicted in Fig.6.

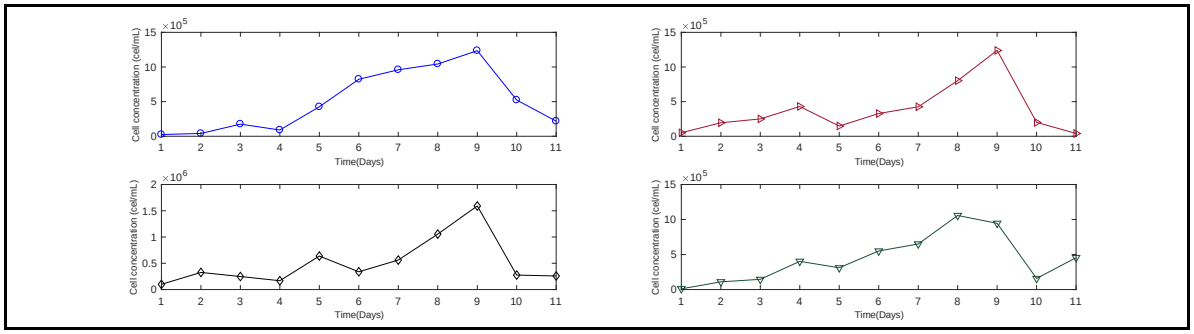

Fig. 6 Crecimiento Poblacional de C. reinhardtii

For the obtained data in the bioassays of the microalgae, analyzes of variance were carried out to compare the experiments. Since the p-value of the F-ratio was 0.8257, (greater than 0.05), there is no statistically significant difference between the means of the 4 bioassays with a 95.0% confidence level. Which suggests that the technique for the culture and growth is suitably handled by laboratory personnel (See fig.7)

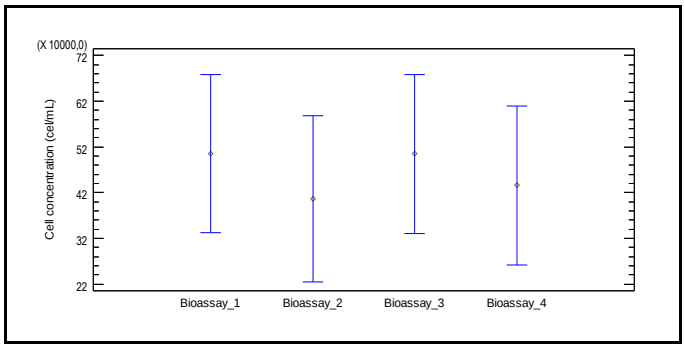

Fig. 7 Growth means comparison for Chlamydomona reinhardtii

Data obtained for optical density in each day of culture of the microalgae Chlamydomona reinhardtii is depicted in Fig. 8.

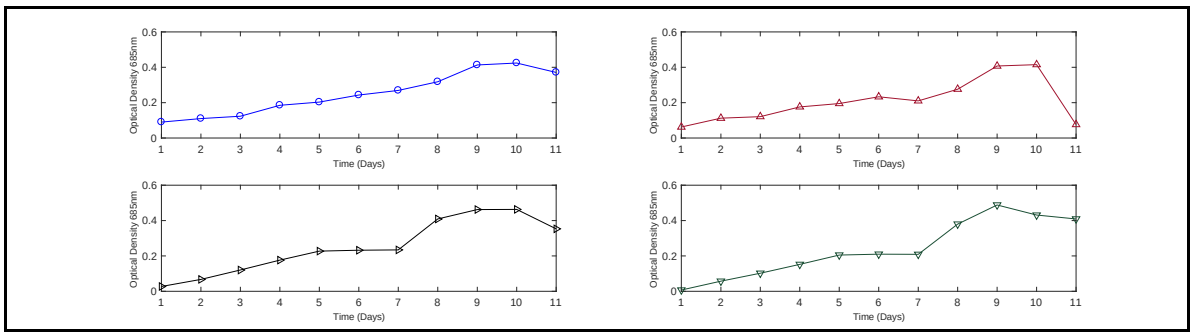

Fig. 8 Optical Density of Chlamydomona reinhardtii

To compare the obtained data and find possible errors in the culture method an ANOVA analysis was performed. The results established a p-value equals to 0.87 which is greater than 0.05, so there is no statistically significant difference between the means of the 4 bioassays with a level of 95.0% of confidence. Fig. 9 corroborates this conclusion as the averages overlap each other, also a lower performance is observed in the bioassay 2.

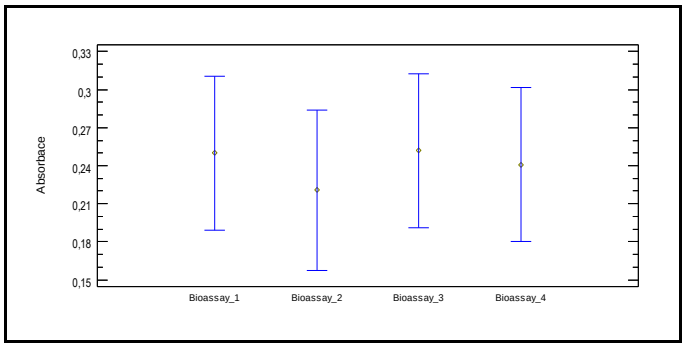

Fig. 9 Comparison of means for optical density of C. reinhardtii

The relationship between optical density and cell concentration of Chlamydomona reinhartii was studied using linear regression, which yielded a p-value equals to 0.0001. The statistics found were: Correlation coefficient® = 0.94, R^2 = 89.3%, R^2 (adjusted) = 87.8%, Durbin-Watson statistic = 1.59. a p-value greater than 0.05 means that exist a statistically significant relationship between the variables. A correlation coefficient of 0.94 indicates a strong relationship between the studied variables. The R^2 statistic indicates that the adjusted model explains 89.3% of the variability in data. Fig. 10 shows the linear relationship between the variables. The adjusted model equation is:

$$Cell\ concentration(cell \cdot mL^{-1}) = 4.29914 \cdot 10^6 \cdot Optical\ density - 398502$$

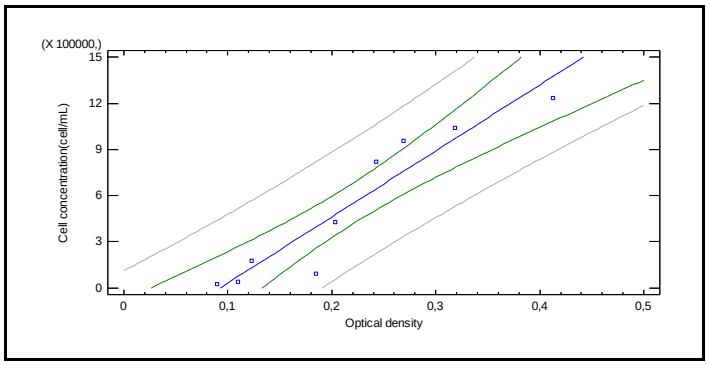

Fig. 10 cell concentration vs optical density for C. reinhardtii

Normality Test for residues using Shapiro-Wilk test yields a p-value of 0.69. due to a p-value greater than 0.05, the idea that the residuals of the linear model were from a normal distribution with 95% confidence was not reject. The Kolmogorov-Smirnov test yields a p-value of 0.98, greater than 0.05, which corroborates the normality assumption of the residues. There is also no indication of a serial autocorrelation in the residuals with a confidence level of 95.0%, since the p-value of the Durbin-Watson statistic is greater than 0.05.

Chlamydomona reinhardtii growth kinetic

The lag phase took place from day zero (0) to day five (5) where the microalgae adjusted to the conditions of the assay. The exponential phase in the bioassays was from day 5 to day 9; the values of growth rate (μ) and the duplication time (td) are shown in Table 2, where the maximum growth rate was reached by the bioassays 1 and 2 (0.26), and also the less duplication time (2.6 days). The maximum biomass production observed for Chlamydomona reinhardtii was 1'590,000 cel • mL-1 reached in third bioassay.

Tabla 2. Growth rate (μ) and duplication time (td) for C. reinhardtii

μ (1/days)	T_d(days)
0,266	2,60
0,264	2,62
0,228	3,04
0,213	3,25

From day 9 death phase began, here growth ceases due to the deficiency of nutrients, the microalgae death is promoted and lasted until the last day of the culture.

Comparison of microalgaes growth

Statistical comparison was made by an analysis of variance (ANOVA) between the means of the data obtained in the tests carried out with each microalga.

The quotient between the estimated between-groups and the estimated within-groups, called the F-ratio, for this case had a value of 11.78 with a p-value of 0.0026. Since the p-value of the F-test is less than 0.05, there is a statistically significant difference between the means of the two cultures (Chlorella and Chlamydomona) with a level of 95.0% confidence (see Fig. 11.).

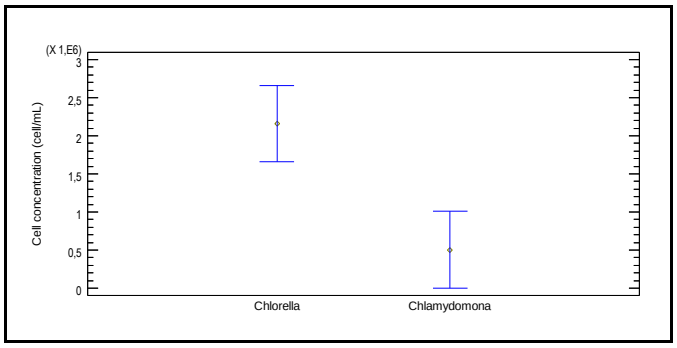

Fig 11. Comparison of culture media of Chlorella and Chlamydomonas.

Contrasting the growth curves of microalgae (Fig. 12) Chlorella vulgaris shows higher biomass yields, obtaining a maximum value of 5'082,500 cells per mL, and Chlamydomona reinhardtii reached a lower Value of 1,590,500 cells per mL, which indicates that, for large-scale biomass production processes, microalgae Chlorella is recommended instead of chlamydomona.

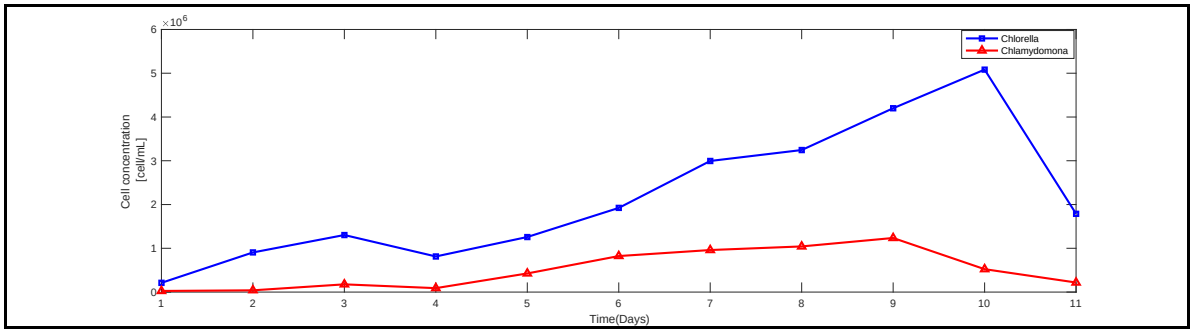

Fig. 12 Comparison of Chlorella vulgaris y Chlamydomona reinhardtii cultures

The comparison of growth rates and duplication times of microalgaes Chlamydomona y Chlorella are depicted in Fig. 13.

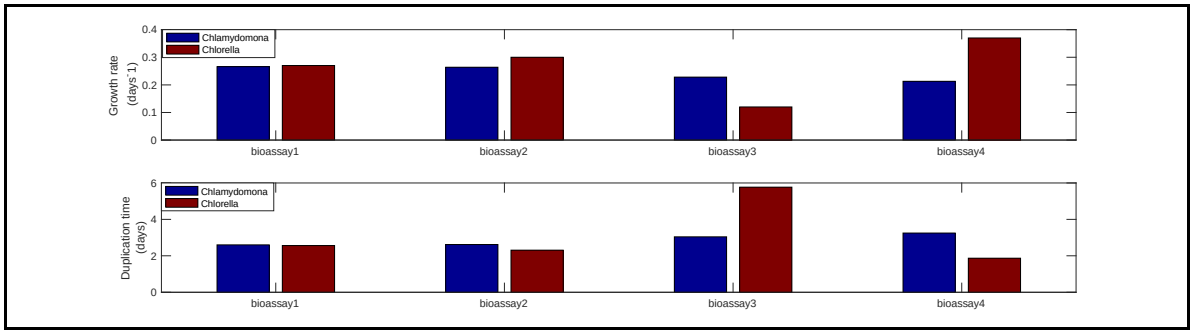

Fig. 13 Comparison of kinetic parameters of Chlorella vulgaris and Chlamydomonas reinhardtii

Chlorella vulgaris presented a higher growth rate in bioassays #1, 2 y 4, but in bioassay #3 it showed an outlier probably due to an error during the cells counting (see Fig. 13)). Plot also showed a lower duplication time in Chlorella vulgaris bioassays, which confirms the higher growth rate of this microalgaes.

In Álvarez et al. the authors found growths of $5x10^6$ cells per mL for Chlorella vulgaris under conditions similar to those of the present study(Álvarez Díaz, Ruiz, J., Arbib, Barragán, J., Garrido-Pérez, & Perales, 2015). This indicates that the cell density for Chlorella is affected by the contribution of CO_2, culture medium and photoperiod. Comparing the data obtained in this study with Wang et al, the authors using different wastewater found values of optical density around 0.5 to 680 nm which is similar to those reported in this work(Wang et al., 2010). Mortensen et al. obtained growths of 5 x 10^6 cells per mL using CO_2 and Harris

culture medium for Chlamydomona reinhardtii, indicating that these factors affect the growth of this microalgae(Mortensen & Gislerød, 2015). Márquez using the modified Chu Nº10 culture medium reached maximum concentrations of 1.9 x 10^6 in Chlamydomonas sp cultures, values that are similar to those obtained in this project bioassays(Márquez & Beccaria, 2006).

Conclusions

Statistical analyzes of the different measurements of cell growth assessed by counting method with neubauer chamber and by optical density with spectrophotometer did not show significant differences between bioassays, indicating that the reproducibility and repeatability of the culture technique is acceptable.

For both Chlorella vulgaris and Chlamydomona reinhardtii, linear regression models were obtained, these may be useful to determine cell number by making optical density measurements by spectrophotometry at 685 nm, which is a simpler method than Neubauer chamber method to count cells.

Chlorella vulgaris showed better kinetic values (growth rate and duplicating time) compared to Chlamydomonas reinhardtii. The amounts of biomass obtained with Chlorella vulgaris make it more favorable in processes where high biomass generation is required, such as the production of biofuels or bioproducts.

In the review of results obtained by different authors on cell density values reached during the same period of time as our bioassays, we conclude that with CO2 supply to crops, biomass production could be increased.

Acknowledgment

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