Growth Optimization of *Chlorella Vulgaris* in Mixotrophic Culture Enriched with Nutrients using Experimental Design

Ildefonso Baldiris-Navarro¹, Jorge Sanchez Aponte¹

Environmental Science Programs, Sena-Cinaflup, Mamonal km4 Cartagena, Colombia.

E-mail:ibaldirisn@sena.edu.co

Abstract: In this research, statistical optimization designs were applied to evaluate the growth of the *Chlorella vulgaris* in mixotrophic cultures enriched with nitrogen and phosphorus. The influence of each factor was evaluated at three levels, so a multifactorial experimental design was created 32. The results obtained showed that microalgae growth improves 32% by adding 50 mg of nitrogen and 25 mg of Nitrogen to the culture. This project demonstrates the relevance of the design of experiments for the optimization of algal biomass production in order to obtain bioproducts.

1. Introduction

The increase in water pollution worldwide due to the discharge of domestic and industrial wastewater, have caused an impact on the quality of water bodies, proven by the increase in the concentrations of nitrogen in the form of ammonium, nitrites, nitrates, and phosphorus in the form of phosphates. The eutrophication of aquatic systems is caused by an excessive concentration of nutrients which alter the characteristics of the food chain and increase entropy in the ecosystem as a consequence of reduced biodiversity, with opportunistic species occupying places previously occupied by other species. Over time, the eutrophication processes might turn a swamp or lake into the mainland, because with the increase in nutrients the amount of plants and animals that live on the surface also increases, which when they die accumulate in the bottom and they are not completely decomposed [1].

In recent years, sewage treatment with microalgae has gained great interest due to its impressive properties. Microalgae are of great ecological importance due to their biodiversity, high growth rates and adaptability to different environmental conditions. They are present in any aquatic environment where that contains a source of carbon, nutrients, sufficient light and appropriate temperatures according to the species. They provide the biosphere with a considerable amount of oxygen for fixing more than 40% of the earth's coal, they are very attractive for producing many products of commercial interest since they can use any residual effluents as a source of nutrients [2]. The use of microalgae, in the treatment of wastewater, has
been the subject of numerous investigations due to its ability to remove significant amounts of Nitrogen (N) and phosphorus (P), heavy metals, fats during its growth and at the same time may accelerate the inactivation of pathogenic bacteria [3]. The wastewater bioremediation capacity of microalgae has already been demonstrated, relevant aspects such as the conversion of nutrients present in algal biomass are described in several researches [4] [5].

The microalgal biomass generated may be used in the production of bioenergetic alternatives such as biodiesel, biohydrogen or methane, in addition animal food, beauty products and pharmaceuticals may also be obtained from it [6] [7] For this reason the production of microalgae using wastewater as cultivation media, may be an alternative for an environmental problem, such as the deterioration of water bodies and also may be a source of different byproducts of great commercial interest in agriculture and industry.

In microalgae cultures, nitrogen is an essential component of many key biomolecules, such as amino acids and nucleotides. Protein synthesis usually depends on an adequate supply of nitrogen to the culture. On the other hand, phosphorus plays an important role in most cellular processes, especially those that are involved in the generation and transformation of metabolic energy, so it is essential for the growth and reproduction of microalgae [8].

Design of experiments (DOE) is a technique that consists of carrying out a series of experiments in which deliberate changes in the variables of a process are induced, in order to observe and identify the causes of the changes in the output response [1]. With this technique may be achieved, for example, to improve the performance of a process and reduce its variability or production costs. Its application in industry includes fields such as chemistry, industrial processes, quality control, etc [9] [10] [11]. The statistical technique of experiment design has been used by several authors in different fields of microalgae use. Skorupskaite et al (2014) used experiment design to optimize a mixotrophic culture of chlorella sp. for the production of biofuel [12]. Priyadharshini et al (2016) used a Plackett-Burman design for the treatment of phenol with Chlorella pyrenoidosa microalgae [13]. Jaafari et al studied the effect of the interaction between the initial concentration of metals, the reaction time and the microalgae dose for the chromium, cadmium and cobalt biosorption by colonial chlorella microalgae [14]. Ido et al applied experimental designs to optimize lipid production of oblique Scenedesmus microalgae assisted with solvent extraction and ultrasound [15].

The aim of this research was to evaluate the growth of Chlorella vulgaris in a medium similar to wastewater enriched with nutrients and determine the concentration of nitrogen and phosphorus for optimal cell growth.

2. Methodology

2.1. Organism and cultivation

Freshwater microalgae chlorella vulgaris was obtained from the biotechnology laboratory of SENA CINAFLUP. Chlorella cells were maintained in modified Conway medium, which contained FeCl$_3$·6H$_2$O (26 g), MnCl$_2$·4H$_2$O (0.72 g), H$_3$BO$_3$ (67.2 g), EDTA (90 g), Na$_3$HPO$_4$·12H$_2$O (40 g), NaNO$_3$ (200 g), Na$_2$SiO$_3$ (40 g), H$_2$O (2 L), traces of metals (2 mL) and solution of vitamins (100 mL). The solution with metal traces contained ZnCl$_2$ (2.1 g), CoCl$_2$·6H$_2$O (2 g), (NH$_4$)$_6$Mo$_7$O$_24$·4H$_2$O (0.9 g), CuSO$_4$·5H$_2$O (2 g) and distilled water (100 mL), and the vitamin solution was composed of decamyl (210 mg) and distilled water (100 mL). The sources of nitrogen and phosphorus for the bioassays were potassium diacid phosphate (KH$_2$PO$_4$) and sodium nitrate (NaNO$_3$).
2.2 Experimental design
To evaluate the microalgae growth cultured in excessive nitrogen and phosphorus, a factorial design $3^2$ was implemented. This factorial design consisted of two factors (added nitrogen and phosphorus) and 3 levels for each factor (0 - 25 - 50 mg, respectively). Table 1 shows the factors and their levels in the experimental design. The design was used to determine the joint effects of several factors on a response variable. It was also used to determine the individual and cumulative effects of these variables and the mutual interactions between them. The complete design consisted of 9 runs, which were performed in duplicate to optimize the levels of selected variables. Data processing and calculations were carried out using a statistical software, Minitab, to estimate the coefficients of the regression equation. The equations were validated by analysis of variance (ANOVA) to determine the significance of each term in the adjusted equations and to estimate the adjustment in each case.

Table 1. Experimental design Factors and levels

| Factors   | levels          |
|-----------|-----------------|
| Nitrogen  | 0 - 25 - 50 mg  |
| Phosphorus| 0 - 25 - 50 mg  |

The statistical model for the design may be written considering the individual effect of each factor and the interaction between them, and is as follows:

$$Y_{ijk} = \mu + \gamma_i + \delta_j + (\gamma\delta)_{ij} + \varepsilon_{ijk}$$

With $i=1,2,3$ $j=1,2,3$; $k=1,...,n$

Where $\gamma_i$ is the effect of the concentration of Nitrogen at its level $i$, $\delta_j$ represents the effect of the phosphorus concentration factor at its level $j$, $(\gamma\delta)_{ij}$ is the interaction effect of both at levels $ij$ and $n$ is the number of repetitions of each treatment. Consequently, the hypotheses to be tested are: $H_0$: $\gamma_i = 0$ (there is no significant effect of the Nitrogen factor over Growth), $H_0$: $\delta_j = 0$ (there is no effect of the Phosphorus factor over Growth) and $H_0$: $(\gamma\delta)_{ij} = 0$ (there is no interaction effect of factors A and B on the response variable). These hypotheses will be tested with the ANOVA; for this, the sums of squares for the three effects included in the previous equation are given by:

$$SC_A = \sum_{i=1}^{3} \frac{Y_{i...}^2}{3n} - \frac{Y_{...}^2}{n3^2}$$

$$SC_B = \sum_{j=1}^{3} \frac{Y_{i.j}^2}{3n} - \frac{Y_{...}^2}{n3^2}$$
Graphically, the design of experiments for the optimization of microalgae growth may be seen in figure 1.

For this model the total sum of squares is calculated with:

\[ SC_T = \sum_{i=1}^{3} \sum_{j=1}^{3} \sum_{k=1}^{n} \frac{Y_{ijk}^2}{n} - \frac{Y_{**}^2}{n^2} \]

and the random error is calculated with the difference:

\[ SC_E = SC_T - SC_{AB} - SC_A - SC_B \]

2.3 Determination of the microalgae biomass and growth rate

Biomass concentration was determined by cell count using neubauer chamber and measuring the optical density at 680 nm. In addition, the dry weight per liter of microalgae was measured day by day, using the total dissolved solids method. A linear relationship was found between dry weight and absorbance at 680 nm.

The specific growth rate was calculated as follows:

\[ \mu (d^{-1}) = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)} \]
Where $X_2$ and $X_1$ are the biomass concentration in ($gL^{-1}$) in $t_2$ and $t_1$ (days), respectively. Duplication time of the cell concentration was calculated using:

$$t_d = \frac{\ln 2}{\mu}$$

3. Results

The biomass concentration (dry weight per liter) of the microalgae cultures was calculated by measuring the solids and the optical density daily. The cell count showed that it was closely related to the optical density at 680 nm and a relationship was found between the two variables according to the following formulation:

Concentración de Biomasa ($gL^{-1}$) = 0.42Abs$_{680nm}$ − 0.22, $R^2 = 0.974$

This relationship is depicted in figure 2

![Figure 2. dry weight vs. absorbance for chlorella vulgaris](image)

The growth of the microalgae with nutrients was made for a period of 8 days, during this time the optical density of the microalgae at 680 nm was measured as well as the dry weight every 24 hours. The variation in cell concentration is shown in Figure 2.
Figure 3. Microalgae growth profiles at different concentrations of N and P.

Population growth of the *Chlorella vulgaris* showed differences in the bio assays carried out with the nutrients. While *Chlorella vulgaris* with 50N-25P concentration reached a population close to 11'800,000 cells / mL, with 25N-50P concentration slightly exceeded 10'000,000 cells / mL (Figure 1) and in the control bioassay did not exceed 8'600,000 cells / mL, which implies a 37.2% improvement in the growth process. The effect of these concentrations may be seen reflected in the growth rates and doubling times (see figure 4). This shows that the best performer was obtained with the mixture of 25 mg of phosphorus with 50 mg of nitrogen.

Figure 4. Growth kinetics for *Chlorella vulgaris*
3.1 Experimental Design

Pareto chart shows the main effects that are those passing the dotted line, in this case are the quantity of nitrogen and phosphorus-nitrogen interaction. These factors affect the growth of the microalgae at the experimental level (see figure 5).

![Pareto chart for Chlorella vulgaris growth. Factor A= N, Factor B= P](image)

Table 2 shows the coefficients of the effects, as well as the analysis of variance. The main effects are those lower than the 0.05 significance level, p-Values (column P), these are shaded in the table.

Table 2. Variance analysis of factors and interaction.

| Source                  | GL | SC Ajust. | MC Ajust. | F-value | p-value |
|-------------------------|----|-----------|-----------|---------|---------|
| Model                   | 8  | 0.62724   | 0.078406  | 8.04    | 0.003   |
| Lineal                  | 4  | 0.16397   | 0.040992  | 4.20    | 0.034   |
| Nitrogen                | 2  | 0.09569   | 0.047845  | 4.91    | 0.036   |
| Phosphorus              | 2  | 0.06828   | 0.034139  | 3.50    | 0.075   |
| interaction of 2 terms  | 4  | 0.46328   | 0.115819  | 11.88   | 0.001   |
| NxP                     | 4  | 0.46328   | 0.115819  | 11.88   | 0.001   |
| Error                   | 9  | 0.08774   | 0.009749  |         |         |
| Total                   | 17 | 0.71498   |           |         |         |

According to these values, the Nitrogen-Phosphorus interaction has a greater significance in the cultivation of chlorella. The mathematical model obtained has an R-quad of 87.73%, which fits well with the experimental data obtained.
Influence of individual factors on cell growth
As may be seen in Figure 6, the nitrogen factor creates an optimal effect at the second level (25 mg) and decays by adding 50 mg. Phosphorus has a similar effect when 25 mg of this is added, but as the dose rises, the growth becomes null and when the is not added, microalgae also has a moderate growth. This indicates that the effect of the added phosphorus is not significant in cell growth as shown by the pareto chart.

Figure 6. Chart of main effects for *Chlorella vulgaris* Growth

The effect of the interactions of the factors on cell growth may be seen in Figure 7. Here it shows that growth is optimal when 50 mg of nitrogen and 25 mg of Nitrogen are added. It may also be noted that as the added phosphorus concentration increases, cell density decreases, which shows that the phosphorus factor has a limit in its concentration, since otherwise the number of microalgae cells decreases.

Figure 7. Interaction plot for *Chlorella vulgaris* growth

The responses of the population of *Chlorella vulgaris* in the different treatments with nutrients of the experimental design reveal that this species is particularly susceptible to the concentration of nitrogen compared to the concentration of phosphorus, since increasing the concentration of the former significantly increases the growth rate, while, although there is also a statistically significant decrease in the values of
these variables due to phosphorus deficiency compared to the control treatment, the results are not as marked and have a limit value of nutrient acceptance, which generates a decrease in the population of the microalgae when it is reached.

Nitrogen deficiency affects growth as it limits the formation of aminoacids that are the building blocks of proteins, which are involved in many aspects of growth, including structural and metabolic processes. If the nitrogen supply is not enough, not enough proteins may be formed to maintain optimal growth levels.

4. Conclusions
The growth of *Chlorella vulgaris* was studied based on the addition of phosphorus and nitrogen using multiple factorial design. It could be observed that the growth is optimal when 50 mg of nitrogen and 25 mg of Nitrogen are added, with this treatment an improvement of 37.2% in the process was obtained. The factor with the greatest influence on the growth variable was the addition of nitrogen and the interaction Nitrogen-Phosphorus.

Acknowledgments

Authors want to thank SENA CINAFLUP y SENNOVA for financing this research.

References

[1] Baldiris-navarro I and Sanchez-aponte J 2018 *Cont. Eng. Sci* 11 1339
[2] Samori G, Samori C, Guerrini F and Pistocchi R. 2013 *Water Res* 47 791
[3] Lananan F, Abdul Hamid S. H, Din W, Ali N, Khatoon H, Jusoh A and Endut A 2014 *Int. Biodeterioration and Biodegradation* 95 127
[4] Roa-Parra A and Cañizares-Villanueva, R. 2012 *Bistua* 10 71
[5] Wang X, Wu Y, Zhang T, Xu X, Dao G and Hu H 2016 *Water Res.* 94 215
[6] Yesica I, Luis A. Ignacio A, Martha P, Paula N, Elvira L, and Hector M 2015 *African J. of Biotech.* 14 1710.
[7] Baldiris-navarro I, Sanchez J and Torres-Virviescas M 2017 *Int. J. of Chemtech* 10 411.
[8] Beuckels A, Smolders E and Muylaert K 2015 *Water Res*, 77, 98.
[9] Miller J N and Miller J C 2010 *Statistics and Chemometrics for Analytical Chemistry*
[10] Montgomery D C, Runger G C and Hubele N F 2009 *Engineering statistics.*
[11] Gutierrez H and de la Vara R 2008 *Análisis y diseño de experimentos*.
[12] Skorupskaite V Makareviciene V and Levisauskas D 2015 *Algal Research*, 7, 45.
[13] Dayana Priyadharshini S and Bakhthavatsalam A K 2016 *Bioresource Tech* 207 150
[14] Jafaari J and Yaghmaeian K 2019 *Chemosphere* 217 447
[15] Ido A L, de Luna M D G, Capareda S C, Maglinao A L and Nam H 2018 *Energy* 157 949