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# Biomass production evaluation of a consortium with prevalence of *Anabaena sp.* in fed-batch cultures with three intensities of irradiance, three levels of nitrate and pilot scaling-up.

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***Abstract: Some cyanobacteria of the genus Anabaena produce compounds of commercial interest. This study reports the effect of the concentration of nutrients in function of sodium nitrate and ammonium nitrate at 0, 2, and 4 mM; and the irradiance at 39, 117 and 234 mol m-2 s-1 over time, on the production of biomass (measured with various methods) from a consortium of microalgae and cyanobacteria with predominant Anabaena sp. in fed batch cultures. Those were scaled up to 10 L and their biomass harvested, dried and chemically analyzed. The growth and biochemical composition of the consortium was influenced by the combination of the above factors, with statistical significance (p<0.05). The best production of pigments and biomass was generated on the combination of factors: nitrate concentration (2 mM) and Irradiance (234 mol m-2 s-1 or 12 klux). A notorious increase in the amount of biomass was produced using ammonium nitrate as a nitrogen source. The biochemical composition of the harvested biomass determined potential applications in the food and pharma industries as well as biofertilizer and soil conditioner.***

***Keywords: Anabaena, biomass, cyanobacteria, irradiance, fed-batch.***

## 1. INTRODUCTION:

The phylum cyanobacteria consists of photosynthetic-oxygenic prokaryotic microorganisms that possess two photosystems (PSII and PSI) and use $H_2O$ as a photoreductive agent in photosynthesis. All known cyanobacteria are photoautotrophic and they mainly use $CO_2$ as a carbon source. Due to a minimal need for nutrients, cyanobacteria can inhabit a wide range of environments even under extreme conditions such as hot springs, desert soils and the poles [1].

Currently, cyanobacteria consist of 2,000 species in 150 genera, with a wide variety of shapes and sizes. With regard to the ecological realm, there are three main groups within the aquatic environment: mat-forming species, bloom-forming species and picocyanobacteria [2].

Ref. [3] points out that cyanobacteria can present different colors: green, blue-green, brown, purple, red, and even almost black. The color of the cells (according to the wavelength range absorbed by their photosynthetic machinery), depends on the types of pigments present and the amounts and proportions in which they are found. A pigment common to almost all of them is chlorophyll a, which is a pigment of the PS I and II reaction centers. Exceptions are known in the case of Prochlorococcus and Acaryochloris. Different types of carotenoids (echinenone, myxoxanthophyll, among others) are commonly present as well. And, with few exceptions, cyanobacteria have phycobilisomes attached to the thylakoid membranes. This is a macromolecular complex composed of phycobiliproteins, which can constitute up to 40% of the soluble proteins of the cell. Allophycocyanin (absorbance maximum 650 nm) is found in the substructure of the nucleus, the part of the phycobilisome that is directly associated with the photosynthetic reaction center. Blue phycocyanin (absorption peak 620 nm), red phycoerythrin (565 nm), and the comparatively rare phycoerythrocyanin (570 nm) are present in rods emanating from the nucleus.

## 2. RESEARCH ELABORATIONS

**Study zone.** This research project was carried out within the Academic Laboratory of the Department of Life Sciences and Agriculture at the University of the Armed Forces - ESPE.

**Obtaining the consortium samples.** The consortium samples were formed by the cyanobacteria *Anabaena sp., Geitlerinema sp., Leptolyngbya sp.* and the microalgae *Chlorella sp.* They were obtained from an inicial consortium constituted by the nitrogen-fixing cyanobacterium *Anabaena sp.* and the microalgae *Chlorococcum sp.* and *Desmodesmus sp.* [4].

**Consortium isolation.** Several liters of $BG_{110}$ medium were prepared in liquid form from four stock solutions and 200 mL were dispensed into three autoclavable glass bottles previously sterilized in an autoclave (MARKET FORGE, STERILMATIC). $BG_{110}$ medium was also prepared in solid form, for which 15 g of Bacto agar was added per liter of liquid $BG_{110}$ medium. From an initial sample, the plates, previously autoclaved, were seeded with solid medium by means of streaking and kept under artificial light until finding evidence of growth, after this, the morphological characterization of the developed colonies was carried out by using an optical microscope with a 40X magnification. The cultures were decanted and homogenized each time it was necessary. The presence of *Anabaena sp., Leptolyngbya sp., Geitlerinema sp.* and *Chlorella sp.* was detected in some colonies of the Petri dishes with solid medium and they were transferred to 10 mL vacutainer tubes, waiting for enough biomass to accumulate for further isolation.

### Laboratory conditions culturing

**First essay.** The growth of the consortium was monitored by content of chlorophyll *a*, carotenoids and phycocyanin, until reaching the stationary phase; in $BG_{110}$ culture media enriched with 0, 2 and 4 mM sodium nitrate (solution $1A/BG_{11c}$) and maintained with constant aeration at 21 ± 2 ° C and unilateral illumination with three light intensities: 39, 117 and 234 μmol $m^{-2}$ $s^{-1}$ equivalent to 2, 6 and 12 klux, respectively. The initial inoculum value was 5 mL in 200 mL of culture.

**Second trial.** To evaluate the effect of a different source of nitrate in the culture medium, a subculture of the first trial was used, which was subjected to an activation process, for approximately two weeks. At that time, in addition to reactivating the strains, it was sought to produce enough biomass to serve as an inoculum for the second test.

The growth of the consortium was monitored by Optical Density (OD), content of chlorophyll *a*, carotenoids and phycocyanin until reaching the stationary phase; in culture media $BG_{110}$ + 2 mM $NaNO_3$ and $NH_4NO_3$, maintained with constant aeration at 21 ± 2 ° C and unilateral illumination at an intensity of 234 μmol $m^{-2}$ $s^{-1}$. The initial inoculum value was 10 mL in 200 mL of culture. The irradiance from fluorescent lamps (LAMPTAN, 110V-10V, 60Hz) was measured using a lux meter (FISHER SCIENTIFIC).

**Determination of growth kinetics**

The results obtained were entered into a spreadsheet (Microsoft Excel), where growth curves were drawn up with respect to the established variables. The "Y" axis corresponds to the growth parameter selected in each experiment; while the "X" axis corresponds to the age of the culture in days. The growth speed (μ) and the doubling rate (Td) were calculated according to the methodology established by Ref. [5].

**Fat-soluble pigment analysis.** 5 mL of previously homogenized culture were taken into properly washed and dried tubes for all replicates of each treatment. It was centrifuged at 4000 rpm for five minutes in a centrifuge (HETTICH, 1810-01 UNIVERSA). 90% of the supernatant was removed with the help of a Pasteur pipette and two mL of methanol were added, the mixture was vortexed (LABNET, VX100) and kept for 24 hours in refrigeration and then centrifuged under the same conditions. The detection of a colorless sediment means that there has been a complete extraction. With the help of a Pasteur pipette, the supernatant was transferred to a 10 mL volumetric cylinder where the volume of the extract was measured. This step was carried out under dark conditions to prevent the photo-oxidation process of chlorophyll.

The extracts obtained were placed in plastic cells for reading in a spectrophotometer (THERMO SCIENTIFIC, GENESYS 20). Absorbance values are 480 nm for carotenoids and 665 nm for chlorophyll *a*, using methanol as a blank. The calculation of the concentration was carried out using the formulas proposed by Ref. [6, 7] for both chlorophyll *a* and carotenoids, respectively.

**Water-soluble pigment analysis.** A 5 aliquot of the culture was centrifuged at 4000 rpm for 10 minutes, then the supernatant was discarded and 2-4 drops of glycerol were added and homogenized. The sample was kept frozen for 30 minutes, then removed from the freezer and 2-4 mL of distilled water were added. The sample was subsequently subjected to successive freezing and thawing processes for later centrifugation at 4000 rpm for 10 minutes. Finally the supernatant was measured in plastic cells using a spectrophotometer at wavelengths 615 nm and 652 nm. Phycocyanin concentrations were determined through the formula proposed by Ref. [8].

**Determination of turbidity.** In the second test, the turbidity of the consortia was evaluated by OD at a wavelength of 750 nm ($OD_{750}$). The readings were done in triplicate. For which, 5 mL of the culture were placed in a vacutainer tube and then the reading was made using the spectrophotometer.

**Determination of dry weight.** To determine the dry weight, aliquots of biomass equivalent to 5 mL were extracted from each experimental unit of the second test, and after measuring their turbidity in triplicate at 750 nm. The value expressed in mg of dry weight / mL was calculated according to the empirical relationship proposed by Ref. [9].

**Culture scaling process.** It consisted of increasing the volume of cultivation to obtain a greater production of biomass. It started using 10 mL vacutainer tubes with 20% in volume of liquid $BG_{110}$ culture medium and unilateral illumination equivalent to 234 μmol $m^{-2}$ $s^{-1}$, subsequently the volume was increased from 200 mL (Stock culture/Assays) to reach 10 L

(Final scaling), taking into account its growth (Optical Density), content of Chlorophyll *a*, Phycocyanin, Carotenoids and Dry Weight with increasing culture volume. Scaling was carried out in glass and plastic containers of different dimensions, which were previously disinfected and sterilized. The whole process lasted for approximately 4 months. To know the estimated values of pigment production, two 5 mL aliquots of the scaled culture were taken five times every three days (50 mL per sampling day, 200 mL in total) for a period of 12 days. The corresponding absorbances were measured the day after taking samples according to established protocols.

Cylindrical geometric similarity was sought to meet the approximate proportions of the photobioreactor tank based on undistorted geometric similarity. The proportionality scale was calculated between the useful vertical lengths of the vessels that were used as a model and the prototype of the final photobioreactor according to the equation presented by Ref. [10]. A 20 L capacity PVC plastic drum for purified water was used as the final container for scaling. Two plastic hoses were introduced into the mouth of the drum, which were directly connected to the air outlets of the compressor.

**Proximal Chemical Analysis of the consortium.** The wet biomass obtained from the consortium's pilot scaling was decanted and harvested. After this, it underwent a solar drying process to obtain dry biomass, on which a proximal chemical analysis was carried out with respect to: moisture, ash, proteins, fats, fiber and total carbohydrates. The analysis was performed by professionals from the CENTROCESAL Cia. Ltda Laboratory located north of the city of Quito-Ecuador.

## EXPERIMENTAL DESIGN

The present research project consisted of two trials: the first applying a $3^2$ Nested Factorial Design and the second a 7x2 Completely Randomized Design. The scheme of both designs is shown in tables 1 and 2.

Table 1. $3^2$ Nested factorial design scheme

**Tiempo** (C1: 5, C2: 11, C3: 18, C4: 25, C5: 28)

| | **Intensidad de luz** | | |
|---|---|---|---|
| **Concentración de Nitrato** | B1: 39 μmol $m^{-2}$ $s^{-1}$ | B2: 117 μmol $m^{-2}$ $s^{-1}$ | B3: 234 μmol $m^{-2}$ $s^{-1}$ |
| A1: 0 mM | A1B1 | A1B2 | A1B3 |
| A2: 2 mM | A2B1 | A2B2 | A2B3 |
| A3: 4 mM | A3B1 | A3B2 | A3B3 |

Table 2. Completely Randomized Design scheme

| Tiempo (C1: 3, C2: 7, C3: 11, C4: 14, C5: 17, C6: 22, C7: 25) | | |
|---|---|---|
| | Fuente de Nitrato | |
| Repeticiones | A: $NaNO_3$ | B: $NH_4NO_3$ |
| 1 | A1 | B1 |
| 2 | A2 | B2 |
| 3 | A3 | B3 |

**Essays.** A first test was proposed to evaluate the interaction effect between nitrate concentration and irradiance on the biomass production of the consortium formed by the cyanobacteria *Anabaena sp., Geitlerinema sp., Leptolyngbya sp.* and the microalgae *Chlorella sp.* in batch cultures; where the pigment content (carotenoids, phycocyanin, chlorophyll *a*) was determined under laboratory conditions exposed to 3 light intensities of 39, 117 and 234 µmol $m^{-2}$ $s^{-1}$ (unilateral illumination) and 3 concentrations of sodium nitrate (0, 2 and 4 mM). To measure these variables, an experimental design was established. Out of it the most effective treatment was selected and the culture was scaled to a biomass production of 10 liters. Similarly, a second trial was established in which the difference in biomass production was evaluated using 2 different sources of nitrate (sodium nitrate and ammonium nitrate) at a concentration of 2 mM and light intensity of 234 µmol $m^{-2}$ $s^{-1}$.

**Analysis and data processing.** The data processing, analysis and calculations were carried out using the Microsoft® Excel® 2011 program for Mac (Version 14.4.2), while the statistical analysis of the results was carried out using the INFOSTAT® program for Windows.

## 3. RESULTS

Isolation of the consortium. As a result of the successive seeding process, around 40 Petri dishes with solid medium were obtained. A wide diversity of new consortia were found, one of them serving as the initial consortium from which the assays could be carried out.

Ref. [11] suggested quantifying the viability or presence of these microorganisms attributing numerical values to them (Table 3).

Table 3. Values assigned to the number of marks for the observation of cyanobacteria and microalgae

| Number of marks | Value |
|---|---|
| 4+ | Abundant |
| 2-3 | Moderate |
| 1 | Escarse |

After approximately 5 months, isolated strains of the *Anabaena sp., Chlorella sp.* and Calothrix sp. genera were obtained. At the end of the second trial, a consortium with predominance of the genus *Geitlerinema sp*. All the aforementioned samples were transferred to 10 mL vacutainer tubes (Figure 1) and donated to the stock of photosynthetic microorganisms of the University of the Armed Forces - ESPE (Figure 2).

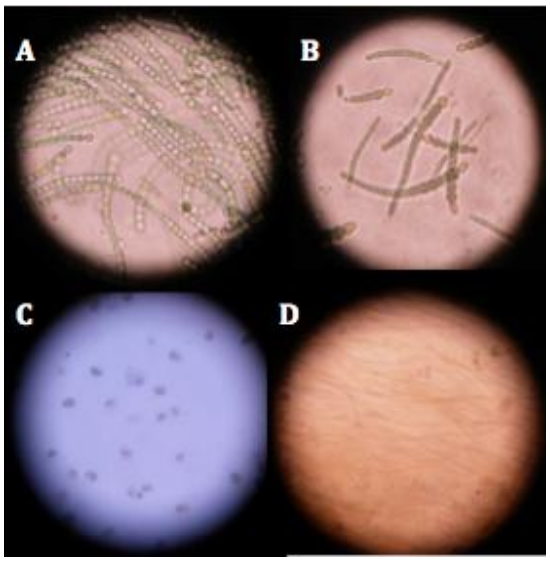


Figure 1. A: *Anabaena sp.* pure, B: *Calothrix sp.* pure, C: *Chlorella sp*. pure, D: Consortium with a predominance of the genus *Geitlerinema sp*. observed at 40X magnification

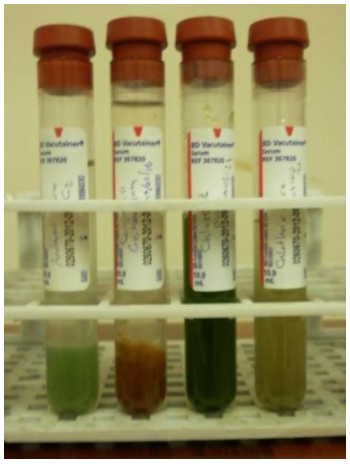

Figure 2. From left to right: *Anabaena sp.* pure, a consortium with a predominance of the genus *Geitlerinema sp.*, *Chlorella sp.* pure and Calothrix sp. pure

**Assay I.** It lasted 28 days. The samples were two aliquots of 5 mL of culture for each experimental unit (27) taken every 6-7 days (270 mL per sampling day, 1350 mL in total). The corresponding absorbances were measured the day after taking the samples according to established protocols.

**Statistic analysis.** The 3 assumptions of the factorial model were not fully fulfilled, presenting high variance values. It was adjusted by applying mixed linear models, from the best fit the analysis was continued.

**Kinetic Analysis.** With the data obtained from the proposed design of experiments, the specific growth speed (μ) and the generation or duplication time (Td) could be defined as the main kinetic parameters. According to the statistical analyzes, it was determined that for the variables Chlorophyll *a*, Carotenoids and Phycocyanin, the best treatment was $A_2B_3$, that is, the one that was subjected to a concentration of 2 mM sodium nitrate and a light intensity of 234 μmol $m^{-2}$ $s^{-1}$ or 12 klux, presenting the maximum production values that are detailed in Figure 3 where a higher production of water-soluble pigments in relation to the fat-soluble ones can be seen, the order is as follows: Phycocyanin > Chlorophyll *a* > Carotenoids.

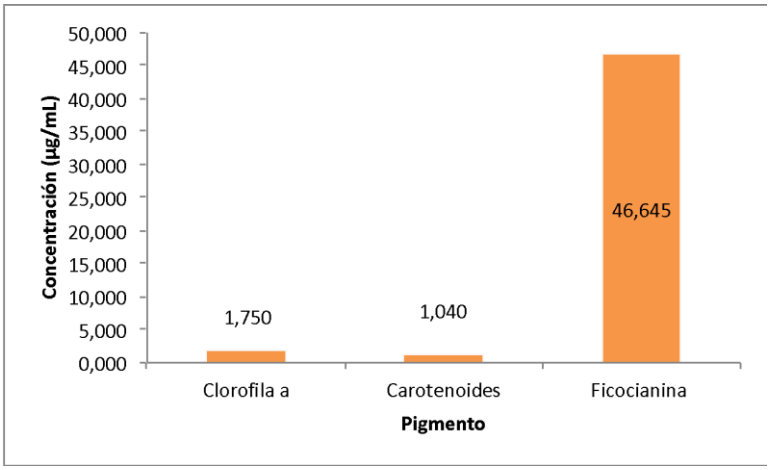


Figure 3. Higher concentrations of pigments produced by the consortium based on the best nitrate and irradiance conditions.

**Assay II.** This trial lasted 25 days. The samples were three aliquots of 5 mL of culture for each experimental unit (6) taken every 3-4 days (90 mL per sampling day, 630 mL in total). The corresponding absorbances were measured the day after taking the samples according to established protocols. As of day 14, a color change began to be noticed in all the experimental units of the test, which went from being blue-greenish (Figure 4B) to yellow-greenish (Figure 4A). This change was accompanied by a decrease in the concentration of phycocyanin and an increase of fat-soluble pigments.

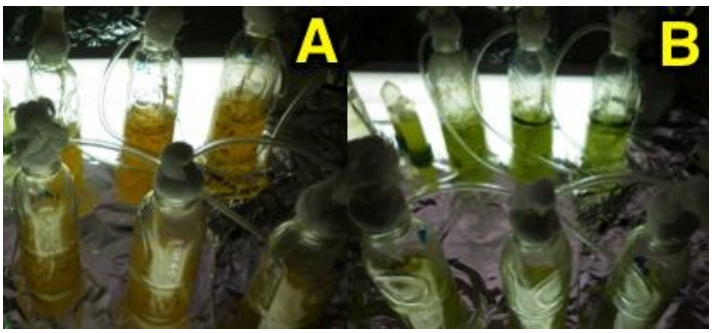


***Figure 4. A: Assay during the second week; B: Assay during the first week***

**Statistic analysis.** The 3 assumptions of the model were met for all variables except for Phycocyanin. Therefore, an alternative was chosen to carry out a transformation of the natural logarithm type. The model was adjusted and the analysis of variance (ANOVA) was applied.

**Kinetic Analysis.** With the data obtained from the proposed design of experiments, the specific growth speed (µ) and the generation or duplication time (Td) could be defined as the main kinetic parameters.

**Escalation Process.** The $n_L$ proportions of geometric similarity between the laboratory scale models and the prototype are detailed in Table 4.

Table 4. $n_L$ proportions obtained during the consortium scaling process

| Dimensions of containers used during scaling | | | $n_L = \frac{Lm}{Lp}$ |
|---|---|---|---|
| **Volume (L)** | **Length (cm)** | **Diameter (cm)** | |
| 0,250 | 6 | 7 | 0,67 |
| 0,50 | 9 | 11 | 0,67 |
| 1 L | 13,5 | 9,7 | 0,69 |
| 3 L | 19,5 | 11 | 0,67 |
| 7 L | 29 | 17,5 | 0,66 |

**Proximal chemical analysis of the consortium.** A summary of the results obtained is shown in Table 5.

Table 5. Results of the Proximal Chemical Analysis performed on the Dry Biomass

| Parameter | Units | Result |
|---|---|---|
| Moisture | %w/dw | 22,02 |
| Protein | %w/dw | 10,25 |
| Fat | %w/dw | 1,9 |
| Ash | %w/dw | 6,6 |
| Fiber | %w/dw | 2,1 |
| Total Carbohydrates | %w/dw | 58,13 |

**Proximal composition.** The proximal composition of the photosynthetic microorganisms present in the collected biomass showed great variability. The protein content found was 10.25% of weight/dry weight, that of lipids was less than 2% w/dw, that of ash reached 6.6% w/dw, only 2.1% w/dw of fiber, while the highest values were 22,02% w/dw moisture and total carbohydrates that represented more than half of its constitution (58.1% w/dw).

## 4. CONCLUSIONS

1. The consortium formed predominantly by the cyanobacterium *Anabaena sp.*, with less presence of *Geitlerinema sp., Leptolyngbya sp.* and the microalgae *Chlorella sp*. was isolated using the streak seeding technique on solid $BG_{110}$ medium.

2. In total, three isolated strains of the genera *Anabaena sp., Chlorella sp., Calothrix sp.* and a consortium with predominance of *Geitlerinema sp.* All of them were donated to the stock of photosynthetic microorganisms of the University of the Armed Forces - ESPE.

3. The growth curves and kinetic parameters showed that a higher production of pigments and biomass was obtained when cultivating the consortium using 234 μmol $m^{-2}$ $s^{-1}$ or 12 klux of Irradiance and a concentration of 2 mM of sodium nitrate.

4. According to the results obtained, it is inferred that the irradiance, concentration and source of nitrate influence the growth and the biochemical composition of the consortium.

5. A noticeable increase in the amount of biomass was produced using ammonium nitrate as an alternative nitrogen source.

6. A greater production of water-soluble pigments in relation to fat-soluble pigments was observed, in the following order: Phycocyanin > Chlorophyll *a* > Carotenoids.

7. The application of the discontinuous culture system fed and scaled-up to a final volume of 10 L allowed the evaluation of the consortium's biomass production.

8. The calculated *nL* ratios between the laboratory scale models and the prototype demonstrated a cylindrical geometric similarity of the undistorted type within the scaling process.

9. The large quantity of phycocyanin phycobiliprotein production that was detected suggested an application within the pharmaceutical industry, as a biofertilizer and as soil conditioner.

10. The chemical proximal composition of the harvested biomass determined a potential application within the food industry.