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Salinity influence on the culture to growth *Porphyridium cruentum* in laboratory conditions

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Abstract
The interest in microalgae is due to their high nutritional value, high productivity, and adaptability to be cultured under laboratory conditions, and was observed that the salinity variable has a significant influence on their growth. *P. cruentum* microalgae stands out for producing commercial bio actives, although limited research on its optimal growth at different salinities was made. This study evaluated the specific growth rate, and duplication rate at 35, 45, 55, 65, and 75 gL^{-1}. The 55 gL^{-1} treatment showed the highest cell growth (50.99x105 cells mL^{-1}). The highest specific growth rates were in the 55 and 75 gL^{-1} treatments (μ=0.167 d^{-1} and 0.164 d^{-1} respectively). The duplication rate was 0.03 days, except for 35 gL^{-1} (0.05 days). The possibility of phased cell growth was observed, underlining the importance of salinity in *P. cruentum* culture for biomass production.

Keywords: *Porphyridium cruentum*, cell growth, environmental factors, salinity, microalgae

1. Introduction
In recent years, interest in microalgae has increased due to their importance in aquatic ecosystems, as the major constituent of primary producers, which were used as food for different stages of aquaculture organisms [1-3]. Microalgae are characterized by high production rates and a great capacity to adapt to different environmental conditions, which allows them to inhabit any aquatic environment that has an adequate source of carbon, nutrients, salinity, and exposure to light [4].
However, under specific culture conditions, microalgae can respond to environmental changes by regulating their metabolites to obtain a higher production of bioactive substances of commercial interest, such as proteins, lipids, vitamins, polysaccharides, enzymes, and pigments, which has generated many applications to obtain biotechnological products such as biofuels, food supplements, bio fertilizers and pigments for the cosmetic or food industry [5,6,7,8]. For this reason, the optimal culture technique was crucial to obtain high growth rates manipulating the environmental variables to increase biomass production in reduced water volume containers and economically viable [9, 10].
Among other factors that affect the growth of microalgae are temperature, light, salinity, pH, and the most important nitrogen, and phosphorus, which allow synthesize chlorophyll and other photosynthetic pigments, nucleic and amino acids, phospholipids, and coenzymes [11]. Similarly, it has been shown that variations in salinity concentration, modified metabolic processes, and cell replication, determine the microalgae nutritional properties, affecting fatty acids and carbohydrate accumulation [12, 5]. However, multiple factors must be taken into account for the growth of any microalgae culture, because changes can produce different biochemical compositions throughout the growth process [13-15].
In this respect, *P. cruentum* has been referenced as a potential source of several high-value commercial chemicals like bioactive substances: Extracellular polysaccharides, polyunsaturated fatty acids, and phycoerythrin during their growing period. In addition, *P. cruentum* can concentrate arachidonic (ARA, 20:4n-6) and eicosapentaenoic (EPA, 20:5n-3) fatty acids, which cannot be synthesized by most other organisms [16]. On the other hand, phycobiliprotein is used as a natural pigment in food, dyes, cosmetics, and fluorescent reagents for diagnostic [17-19].

That's why the mean goal of this study was the management of different salinity concentration to produce big quantities of *P. cruentum* to make subsequently, in other study, the extraction of another chemical substances.

P. cruentum can grow in a wide range of salinity and pH and is found to be able to grow in culture media made from artificial seawater [11, 20]. However, in recent years, research on culture conditions of *P. cruentum* has only focused on the production of bio actives such as phycoerythrin, polysaccharides, and fatty acids [5], but the responses to different factors such as salinity for optimal growth have not been particularly studied.

Therefore, the objective of the present research was to evaluate the effect of different salt concentrations on the growth of the microalgae *P. cruentum* to determine the optimal production range.

2. Materials and Methods

2.1 *P. cruentum* culture

The microalgae were acquired from the microalgae ceparium of the Live Food Production and Biofloc Laboratory at the Universidad Autónoma Metropolitana, Unidad Xochimilco, from an isolated sample cultured in solid agar plate in 500 mL flask, with 45 gL-1 until reaching a concentration of 34,500 cells mL-1 to be used later as initial inoculum.

2.2 Experimental design

For *P. cruentum* culture, 18 L plastic containers with five different salinity concentrations (35, 45, 55, 55, 65, and 75 gL^{-1}) with three replicates per treatment were used. The containers were inoculated with 34,500 cells mL^{-1} and were kept under a warm light source produced by an LED bulb supplying an irradiance of 50 µmol $m^{-2}s^{-1}$ [21], with light/dark periods of 12 h, with continuous aeration to prevent microalgae precipitation. The pH was maintained at 7.5 [11] and temperature of 25± 2 °C [22].

Every third day, the culture medium was fertilized with 0.5 mL of Triple17 (17% N-P-K, VIGORO excelso®) and 1.0 mL of a commercial multivitamin (vitamins A, B_1, B_2, B_6, B_{12}, C and D_2) nicotinamide; calcium; phosphorus; iron; magnesium; zinc; manganese; potassium and soy lecithin; BIOFARMA CMD, SA DE CV). To avoid salinity fluctuation at experimental treatments, this variable was also measured every third day with an AO refractometer (0-100 units).

2.3 Cell density

Cell density was evaluated for 30 days every third day, with a 0.1 mm deep Neubauer chamber (LUZERN® PRO1001033) with a Leica ICC50 HD microscope connected to the imaging and counting program (Image® Pro-Plus 7.0, Media Cybernetics). With these data, a growth curve from each experimental salinity was made. The kinetic parameters selected were the specific growth rate (µ) and duplication rate (DR), using the equations described by Guillard (1973) [23].

2.4 Specific growth rate (µ)

Specific growth rate (µ) was obtained with the formula:

$$\mu = \frac{Ln(N_2) - Ln(N_1)}{t_2 - t_1}$$

Where,

µ = Specific growth rate in days

N1= Number of cells at time T1

N2= Number of cells at time t2

T1 and T2= Initial and final time in days

2.5 Duplication rate (DR)

The following formula was used to obtain the duplication rate (DR)

$$DR = \frac{In\ 2}{\mu}$$

2.6 *P. cruentum* biomass

At the end of the experiment, the biomass of all cultures was obtained in dry, freeze-dried, and frozen weight.

2.7 Statistical analysis

With all data, a one-way ANOVA analysis was made to observe significant differences ($P<0.05$) between experimental treatments. When a significant difference was obtained for this statistical method, a mean multiple analysis test was made using Turkey's method.

3. Results

3.1 Cell density

Cell density mean values (±D.S.) of *P. cruentum* are shown in Table 1. The highest value was found at 55 mL^{-1} experimental salinity with $50.99x10^5$ cells mL^{-1}, followed by 75 mL^{-1} experimental salinity with $47.57x10^5$ cells mL^{-1}. Meanwhile, 45 mL^{-1} experimental salinity only obtained $20.17x10^5$ cell mL^{-1}.

Table 1: Mean cell 10^5 mL^{-1} values of *P. cruentum* (±D.S.) from five experimental treatments

Culture days	Experimental treatments (cel x 10^5 mL^{-1})				
	35gL^{-1}	**45gL^{-1}**	**55gL^{-1}**	**65gL^{-1}**	**75gL^{-1}**
0	0.34±2340	0.34±1029	0.34±1157	034±1011	0.34±2419
2	0.76±739	1.34±1331	3.39±1776	1.87±1462	3.17±1826
4	1.64±1807	2.68±2258	6.79±812	3.75±2049	6.34±2189
6	2.62±1667	4.03±2068	10.19±2098	5.63±1576	9.51±1971
8	3.73±1920	5.37±1835	13.59±1269	7.51±1324	12.68±1512
10	4.94±2213	6.72±1164	16.99±992	9.39±2113	15.85±1535
12	6.27±2094	8.06±2544	20.39±1934	11.27±2582	19.03±2267
14	7.70±2485	9.41±1446	23.79±953	13.15±1150	22.20±974
16	9.26±2045	10.75±2397	27.19±1839	15.03±849	25.37±1551
18	10.92±2027	12.10±1208	30.59±1631	16.91±697	28.54±2160
20	12.70±2577	13.44±1188	33.99±2139	18.79±975	31.71±1310
22	14.58±1003	14.79±902	37.39±1219	20.67±872	34.89±1153
24	16.58±872	16.13±2343	40.79±2120	22.55±1053	38.06±1344

26	18.70±978	17.48±2076	44.19±778	24.43±2241	41.23±1085
28	20.92±1607	18.82±1511	47.59±2243	26.30±1622	44.40±1846
30	23.26±2282	20.17±1788	50.99±2258	28.18±2094	47.57±799

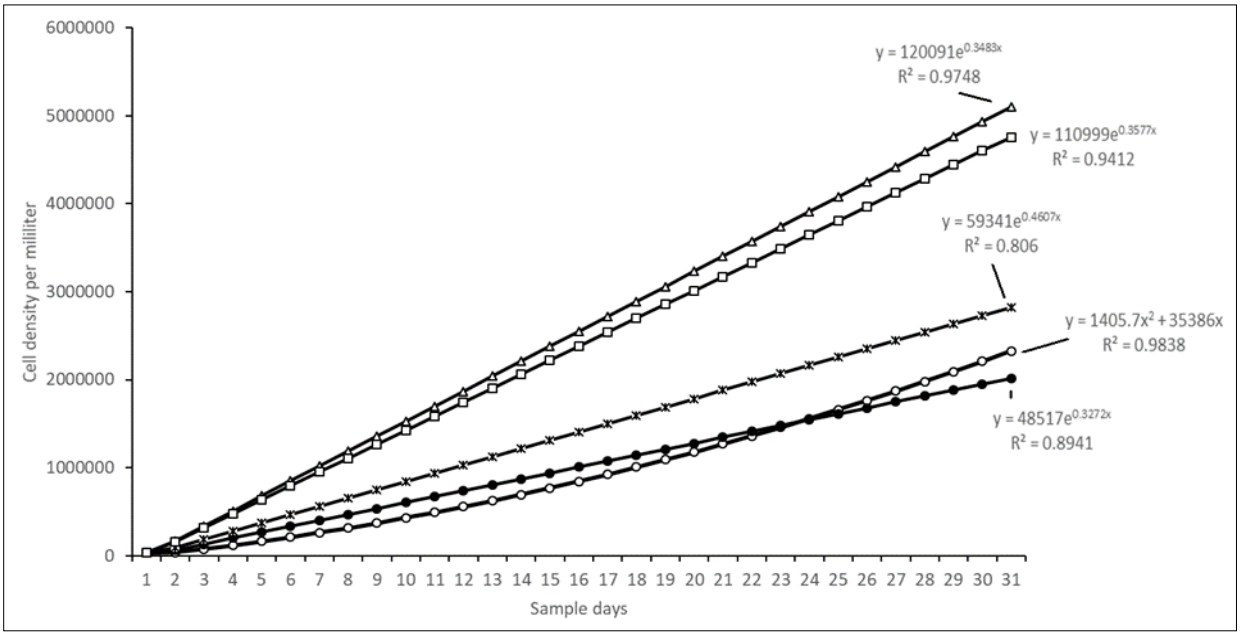

Fig 1: Cellular per milliliter density growth curves from the five experimental salinity treatments with *P. cruentum*.

Fig-1 Shows the growth curves of *P. cruentum* cellular density. ANOVA test shows significant differences (p<0.05) and the Turkey test shows that all experimental treatments have significant differences between them (p<0.001).

3.2 Cellular growth kinetic

All values per experimental treatment are shown in Table 2. The highest values of specific growth rate were shown in 55 y 75 gL^{-1} treatments with 0.167 d^{-1} and 0.164 d^{-1} respectively. The lowest value was shown in 45 gL^{-1} treatment with 0.136 d^{-1}. The ANOVA test shows significant differences between 45 gL^{-1} y 55 gL^{-1} treatments (P=0.0016), also, between 45 gL^{-1} and 75 gL^{-1} treatments (P=0.0262), and between 55 gL^{-1} and 75 gL^{-1} treatments (P=0.0027).

Regarding the duplicate rate at experimental treatments, it can be observed that all treatments take 0.03 days to duplicate their quantity except the 35 gL^{-1} experimental treatment which takes 0.05 days to duplicate (Table 2).

Table 2: Specific growth rate (μ) and duplicate rate (DR) of the five experimental treatments

Experimental treatment	(μ)	DT
35 gL^{-1}	0.140	0.05
45 gL^{-1}	0.136	0.03
55 gL^{-1}	0.167	0.03
65 gL^{-1}	0.147	0.03
75 gL^{-1}	0.164	0.03

3.3 *P. cruentum* biomass

At the end of experimental treatments was obtained 280 liters of *P. cruentum* of which 52 g were in dry weight form, 41.72 g of lyophilized form, and 90 mL in freezing form.

4. Discussion

Among the main factors that modified the quality and cellular quantity of microalgae are temperature, irradiance, pH, and nutrient source [13-15]. During this research, temperature, pH, and irradiance applied to all experimental treatments were maintained constantly and there were maintained within at optimal range reported for *P. cruentum* culture medium [22, 11, 21], and thus did not directly influence the microalgae growth.

The variable salinity was an important factor affecting marine microalgae growth [24]. Salt stress causes a different physiological and biochemical response, such as photosynthesis inhibition, synthesis of secondary metabolite, and osmoregulation [25]. These microalgae responses are differentiated according to salinity adaptability and their tolerance capacity [26]. The *P. cruentum* is a marine microalga so that make culture medium at low salinity could significantly growth and cell morphology changes [5,27], which was confirmed by the experimental results in this research, because *P. cruentum* grows slowly under low salinity conditions ($20.17x10^5$ cells mL^{-1}). In comparison with Saracco-Alvarez [28] study, which uses the Guillard f/2 culture medium in their *P. cruentum* cultures, obtained better values (100% more) of cell density with $44.51x10^5$ cells mL^{-1}.

In this research, it was observed that *P. cruentum* showed a decrease in cellular number at 24 culture days when it was exposed to 45 gL^{-1} culture salinity. However, under that salinity (35 gL^{-1}) cell growth was higher after that day. The same results were shown in Kim *et al.* [29] study, when they made a comparison study of bioethanol production with *P. cruentum*, both in marine and freshwater culture medium, found that it can be obtained the maximum biomass production only considering the specific time of microalgae harvest. Therefore, it is suggested that the goal of producing the highest cell concentration can be achieved by using the phased culture time technique. Culture in high salt concentrations can be used as a quick method to grow and concentrate *P. cruentum* cells at the initial culture phase and low salt medium concentrations, can maintain the time survival of *P. cruentum* cells which allows the increase and accumulate biomass at the subsequent culture time [5].

The differences in cellular growth in *P. cruentum* showed in this experiment (low density at 65 gL^{-1} with 28.18 x 10^5 cell mL^{-1}; and highest density at 55 gL^{-1} with 50.99 x 10^5 cells

mL^{-1}) are due mainly to exposure to salt stress which cause water loss, ionic imbalance, reduced photosynthetic rate, and overproduction of reactive oxygen species [30]. However, a cell concentration of 47.57 $x10^5$ cells mL^{-1} is shown in the experimental treatment of 75 gL^{-1} salinity. Contrasting these results with Manandhar-Shrestha *et al*. [31], who analyzed the response of photosynthesis to salinity stress in *Porphyridium* sp, found that cells can acclimatizing to ionic stress. This can observed for their ability to establish a new growth phase after a short lag period.

However, the duplication rate did not have changes concerning salinity variation at 45, 55, and 75 gL^{-1} experimental treatments, only 35 gL^{-1} experimental treatment shows a duplicate rate of 0.02. This finding corroborates that Lopez *et al*. [32] mentioned that high duplication rate values show low specific growth rates. Other authors like Saracco-Alvarez (2007) [28] found values of $\mu = 0.55\ d^{-1}$ and DR= 1.26 with *P. cruentum* cultured at 35 gL^{-1}. The value differences with our findings can be due to the composition of the culture medium, because Saracco-Alvarez [28] uses Guillard f/2 culture medium and the mixed nutrients were not a constraint to is growth. You and Barnet [33] obtained values of $\mu = 0.38\ d^{-1}$ and DR= 2.66 days, cultured in a BIO III bioreactor, which could be attributed to the environmental conditions and the type of culture. Razaghi *et al*. [34], obtained growth rates between $\mu = 0.05$ and DR = 0.2 d^{-1} when comparing the effects of nitrogen on growth and carbohydrate formation of *P.cruentum*.

Although these values were different in this research, it is complicated to compare the results because the culture methods, the system used, and culture conditions like salinity and light availability were different and can modify the microalga growth density [35].

5. Conclusions

P. cruentum shows better growth values at laboratory conditions at 55 gL^{-1}. However, phased cell growth is possible using different salt concentrations. Since this microalga has a commercial interest in the chemical products that can be synthesized, it is essential to consider the optimal combination of culture conditions in relation to the salinity to maximize the production of compounds of interest, as well as a high cell density.

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