Cellular viability and growth of microalgae: effect of the culture medium
Crecimiento y viabilidad celular de microalgas: efecto del medio de cultivo

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Key words:
alternative culture media; cryopreservation; fertilizer; methanol; nutrients

Abstract

In Aquaculture, the use of microalgae is fundamental in the first feeding of native fish species, since their optimal nutritional level favors survival. Successful production of microalgae under laboratory conditions depends on the culture medium used during the cryopreservation stage of production. Agricultural fertilizers can be used as a low-cost alternative culture media that promotes cell growth and cryopreservation. The objective was to evaluate the effect of two culture media on population growth (PG) and post-thawing viability (PTV) of three microalgal species (*Chlorella* sp., *Desmodesmus* sp., and *Ankistrodesmus* sp.). The PG and PTV were evaluated for F/2 Guillard and Nutrifoliar® culture media. Instantaneous growth rate (K), doubling time (dt), yield (y), and maximum density (md) were evaluated for PG in both culture media. For VCP, 5 and 10 % methanol was used in six treatments. The PTV was classified as no cell damage (NCD), cell damage (CD), and marked lesions (ML). Population growth did not differ among microalgae ($p > 0.05$). T1 resulted in the lowest dt for *Desmodesmus* sp., ($p < 0.05$). T2 showed the highest y and md for the three microalgae ($p < 0.05$). Regarding post-thawing cell viability, the highest NCD for *Chlorella* sp. at day (d) 0 was similar between T3 and T4, and at d 5 it occurred in T6; for *Desmodesmus* sp., at d 0 it occurred in T6, and at d 5 it was similar between T6 and T1; for *Ankistrodesmus* sp., at d 0 and d 5 it occurred in T3. It is concluded that the culture medium Nutrifoliar®, is a viable alternative and of low cost for the culture and the cryopreservation of microalgae of fresh water.

Palabras clave:
medios alternativos; criopreservación; fertilizante; metanol; nutrientes

Resumen

En acuicultura, el uso de microalgas es fundamental en la primera alimentación de especies nativas de peces, pues su óptimo nivel nutricional favorece la sobrevivencia. El éxito de la producción de microalgas depende, entre otras, del medio de cultivo empleado. Los fertilizantes agrícolas usados como medio de cultivo son una alternativa de bajo costo que favorece el crecimiento celular y la criopreservación. El objetivo fue evaluar el efecto de dos medios de cultivo sobre el crecimiento poblacional (CP) y la viabilidad celular post-descongelación (VCP) de microalgas *Chlorella* sp., *Desmodesmus* sp., y *Ankistrodesmus* sp. Se evaluó el CP y VCP los medios de cultivo F/2 Guillard, y Nutrifoliar®. Para el CP en ambos tratamientos se determinó: crecimiento (k), tiempo de duplicación (td), rendimiento (r) y densidad máxima (dm). Para VCP se empleó metanol al 5 y 10 %, en seis tratamientos. La VCP se clasificó: sin daño celular (SDC), daño celular (DC) y lesiones marcadas (LM). El crecimiento poblacional fue igual para las tres microalgas ($p > 0,05$). El T1 tuvo el menor td para *Desmodesmus* sp ($p < 0,05$). El T2 presentó el mayor r y dm para las tres microalgas ($p < 0,05$). En la viabilidad celular post-descongelación, el mayor porcentaje SDC para *Chlorella* sp., al día (d) cero, fue similar en T3 y T4 y al d cinco fue en T6; para *Desmodesmus* sp, al d cero fue en T6 y al d cinco fue similar en T6 y T1; mientras que, para *Ankistrodesmus* sp, al d cero y cinco se presentó en T3. Se concluye que el medio de cultivo Nutrifoliar®, es una alternativa viable y de bajo costo para el cultivo y la criopreservación de microalgas de agua dulce.

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Introduction

In aquaculture, the use of microalgae is necessary for the first feeding of native fish species (Luna and Arce, 2017; Alam *et al.,* 2020). Microalgae have adequate levels of nutrients, which help increase fish survival. Microalgal nutrients are used directly and indirectly by fish larvae in the food chain (Kiron *et al.,* 2016; Sharifah *et al.,* 2016; Sipaúba *et al.,* 2017). Microalgae *Chlorella* sp., *Desmodesmus* sp. and *Ankistrodesmus* sp. stand out for their nutritional value. These species provide protein (16 to 60 %), carbohydrates (14 to 22 %) and lipids (12 to 17 %), as well as vitamins, amino acids, and saturated and unsaturated fatty acids (Sharifah *et al.,* 2016; Sipaúba *et al.,* 2017; Rinanti and Purwadi, 2017; Soares *et al.,* 2017). Furthermore, microalgae show rapid growth and high cell density when nitrogen (N), phosphorus (P), and potassium (K) levels are adequate in the medium, (Sipaúba *et al.,* 2017; Shatwell and Köhler, 2019). Nitrogen in its assimilable form, such as nitrate (NO_3^-) or ammonium (NH_4^+), is one of the most important nutrients for microalgal growth (Shatwell and Köhler, 2019; Nagao *et al.*, 2019). Thus, successful development of microalgal biotechnology relies on culture media that optimize algal growth and nutritional value (Muñoz *et al.,* 2012). However, the culture medium is one of the main limitations, since it requires expensive, analytical-grade reagents (Ortiz *et al.,* 2012). For this reason, several researchers have proposed using low-cost alternative media, such as agricultural fertilizers with adequate N: P: K proportions (Ortiz *et al.,* 2012; Hernández and Lebbé, 2014; Silva-Benavides, 2016; Nagao *et al.,* 2019), to obtain similar or higher microalgal biomass production in comparison with traditional media (Jad, 2012; Rahardini *et al.,* 2018; Shatwell and Köhler, 2019).

The culture medium ensures growth, productivity, and concentration of chlorophyll, proteins, and carbohydrates in microalgae (Silva, 2016), while environmental conditions (e.g., light) determine its physiological state and biochemical composition (Vásquez *et al.,* 2013; Allam *et al.,* 2020). Keeping adequate nutrient proportions is vital for microalgal growth, otherwise concentrations of reactive oxygen species increase, affecting the DNA, telomeres, membrane lipids, as well as proteins and carbohydrates in organelles (Ríos, 2003; Benson and Bremner, 2004; Fujita *et al.,* 2006; Jeyapalan and Sedivy, 2008; Bhattacharya and Goswami, 2020). Antioxidants provide protective mechanisms to counteract the effects of free radicals, (Bumbak *et al.,* 2011). Adequate concentration of assimilable nutrients in the culture medium increases cellular resistance by stimulating structural and functional protection mechanisms against variations in temperature, light, and/or mechanical processes such as centrifugation, and freezing and thawing used in cryopreservation processes.

Cryopreservation is a biotechnological technique to preserve cell structures or biological material at low temperatures, which inactivates physiological processes for a period of time (Day and Brand, 2005; Smith *et al.,* 2008; Bui *et al.,* 2013; Saadaoui *et al.,* 2016). Cryopreservation optimizes production, maintenance, and genetic stability of microalgae (Day and Brand, 2005; Bui *et al.,* 2013; Aray-Andrade *et al.,* 2018). The main challenge in cryopreservation is to develop techniques to guarantee post-thawing cell viability. Scarce reports in the literature describe the effects of the culture medium used prior to cryopreservation on the viability of cells after thawing. The present study evaluated the effects of two culture media on population growth and subsequent post-thawing cellular viability of freshwater microalgae (*Chlorella* sp., *Desmodesmus* sp. and *Ankistrodesmus* sp.).

Materials and methods

The study was conducted in the Live-Food Laboratory of San Silvestre Fish Farm (LAVPSS) in Barrancabermeja, Colombia. *Chlorella* sp., *Desmodesmus* sp., and *Ankistrodesmus* sp. were isolated from ponds at the fish farm by manual micropipetting, under laboratory conditions (temperature, light, among others). Maintenance of the obtained strains was carried out following the techniques of serial replication and successive dilution, as well as monoculture in Petri dishes using F/2 (Guillard and Ryther, 1962) as nutrient medium.

The three microalgae were grown batchwise under aseptic conditions with sterile, nourishing water, starting from test tubes of 10 mL to translucent glass units of 150 to 500 mL. Temperature (24 °C), light (24 hours/day, with fluorescent 1350 lumens E-TLT818G13P 18W led lamps), and aeration (plastic hose, 5.0 mm diameter) were kept constant during the experiment.

This descriptive and experimental study was conducted in two stages to evaluate population growth and cryopreservation of three freshwater algae in two culture media.

Population growth of microalgae

All microalgae were cultivated in translucent glass units (500 mL useful volume). The experimental treatments consisted of two culture media: F/2 (Guillard and Ryther, 1962) (T1-F/2) or a

commercial fertilizer (Nutrifoliar® Complete, Colinagro S.A, Colombia) (T2-NUT), composed of major elements including total nitrogen (200 g/L), P2O3 (100 g/L), K2O (50 g/L); secondary elements including MgO (10 g/L), S (14 g/L) and micronutrients including B (1.5 g/L), Cu (2.5 g/L), Fe (1.0 g/L), Mn (1.0 g/L), Mo (0.03 g/L) and Zn (5.0 g/L). Three replicates were made of each treatment. The fertilizer was prepared by diluting 0.99 mL NUT in 500 mL sterile water.

Three aliquots were counted every 24 hours per experimental replicate using a Neubauer camera (1/10 mm deep, Bright line-Boeco, Germany) and an optical microscope (Leica DM 500, USA). The following population parameters were established in the cultures: instantaneous growth rate (*k*), doubling time (*dt*), yield (*y*), and maximum density (*md*).

Post-thawing cell viability

The microalgae were previously cultured in test tubes with 9 mL water and one of the culture media (F/2 or NUT) and subsequently cryopreserved to evaluate the effect of the culture medium on post-thaw viability (PTV) on days (d) 0 and 5.

Microalgal cryopreservation followed the protocol of microalgae and fish semen cryopreservation developed at the Fish Research Institute of Universidad de Córdoba, Colombia (CINPIC). Methanol (MET; 5 % or 10 % v/v) was used as cryoprotective agent combined with F/2 or NUT in four of the treatments frozen, as follows: F/2-5 % (T1), F/2-10 % (T2), NUT-5 % (T3), NUT-10 % (T4), and two treatments that did not include methanol (WOC): F/2-WOC (T5), and NUT-WOC (T6). One mL of the pre-frozen mixture containing 20 % of the concentrated microalgae + 80 % of 5 or 10 % MET was prepared at room temperature (23 °C) in a 2 mL Eppendorf. The biological material per treatment was packed in four 0.5 mL unsealed straws, using insulin syringes and 100 µL pipette tips.

The cryopreservation protocol was developed in three stages: equilibrium, freezing and thawing. The equilibrium stage lasted 30 minutes, in the dark, at room temperature. The straws were submerged in a nitrogen vapor tank (dry shipper, -80 °C approximately) for 30 minutes and then stored in a tank of liquid nitrogen (-196 °C) for 35 hours. Thawing was done in a water bath (35 °C for 90 seconds). The thawed microalgae were inoculated in test tubes with 9 mL of sterile water enriched with the same medium previously used for the culture (F/2 or NUT). The cryoprotectant was then removed by centrifugation (3500 rpm for 10 minutes), the supernatant was removed and the concentrated microalgae in new tubes were inoculated with the culture medium of each treatment at room temperature. Four replicates were made for each thawed treatment, inoculating 1 mL into four tubes under equal conditions.

Cell viability was evaluated with the following criteria: 1) No cell damage (NCD): cells have well-defined shape, vibrant green color, complete cytoplasm, and well-formed cell wall (*Chlorella* sp. and *Desmodesmus* sp. present defined pyrenoid, while *Desmodesmus* sp. and *Ankistrodesmus* sp. have visible chloroplasts and vacuoles); 2) Cell damage (CD): cells have contracted cytoplasm, undefined nucleus, opaque color (*Desmodesmus* sp. and *Ankistrodesmus* sp. have non-visible chloroplasts and *Desmodesmus* sp. have irregular seta); 3) marked lesions (ML): deformed cells, contracted cytoplasm, cell wall rupture, and non-visible or undefined pyrenoid (*Chlorella* sp. and *Desmodesmus* sp.).

Experimental design and statistical analysis

Population growth and cryopreservation of the three microalgae was conducted under a completely randomized experimental design. Two treatments, with three replicates per treatment, were used to evaluate population growth. Cryopreservation was assessed with 24 experimental replicates in six treatments, with four replicates per treatment.

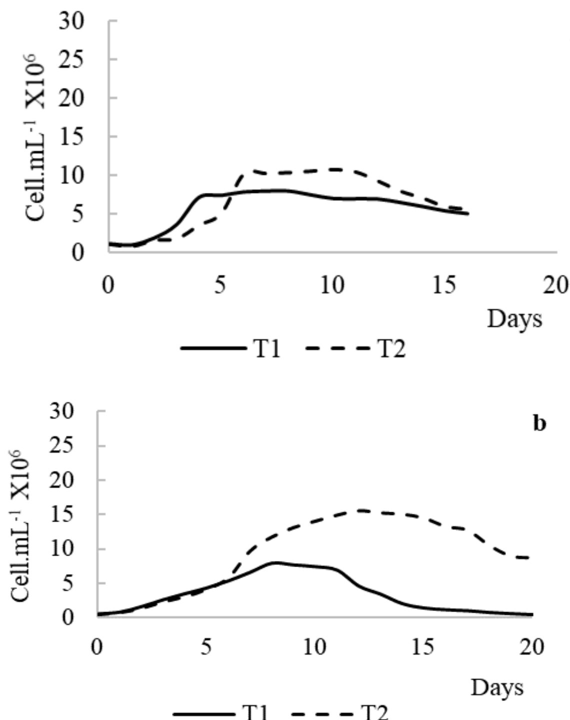
Data were subjected to normality and homogeneity of variance tests. Values are expressed as mean ± standard deviation. Data were analyzed by means of ANOVA, and Tukey Multiple Range test or nonparametric analysis of Kruskal-Wallis was applied when a significant difference was observed. In all cases a 95 % confidence interval was assumed ($p < 0.05$). Statistical analysis was performed with the IBM SPSS® Statistics software, version 23.

Results

Population growth

In culture, the three microalgae presented different population curves (Figure 1a,b,c). *Chlorella* sp. had a shorter cultivation period (16 d) and lower density (Figure 1a,b,c), with exponential growth phase occurring between d 2 and 4 in T1 (F/2), and between d 2 and 6 in T2 (NUT). Its maximum density occurred at d 8 in T1 and d 10 in T2. *Desmodesmus* sp. presented a 20-d cultivation period for both treatments (Figure 1), and its exponential growth occurred from d 1 to 6 in both treatments. Its maximum density was recorded in d 8 and d 13

for T1 and T2, respectively. *Ankistrodesmus* sp. presented the highest cultivation period (25 d) and greater density for both treatments (Figure 1a,b,c). The exponential growth phase occurred from d 2 to d 12 in T1, and from d 3 to 13 in T2. The maximum density was recorded on d 12 (T1) and d 13 (T2).



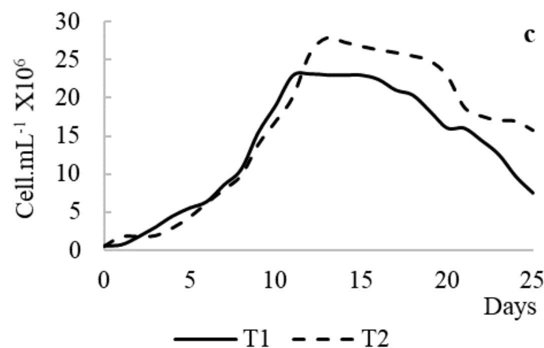


Figure 1. Growth of three freshwater microalgae populations in two culture media: a) *Chlorella* sp. b) *Desmodesmus* sp. c) *Ankistrodesmus* sp. T1: treatment 1 (F/2 medium (Guillard and Ryther, 1962)). T2: treatment 2 (Nutrifoliar Complete medium).

Regarding population parameters of *Chlorella* sp., k and dt did not differ between treatments ($p > 0.05$), while y and md values were higher in T2 ($p < 0.05$) compared to T1 (Table 1). For *Desmodesmus* sp., k showed no difference between T1 and T2 ($p > 0.05$), but the other population parameters presented differences ($p < 0.05$), with lower dt, y and md for T1 compared to T2 (Table 1). As for *Ankistrodesmus* sp., no differences in k and dt were observed ($p > 0.05$; Table 1); in contrast, y and dm were higher in T2 ($p < 0.05$; Table 1).

Table 1. Population parameters of three microalgae (average ± SD) in two culture media. T1: treatment 1 (F/2 medium (Guillard and Ryther, 1962)). T2: treatment 2 (Nutrifoliar Complete medium). k: instantaneous growth rate. dt: doubling time. y: yield. md: maximum density. Different letters in the same row indicate differences between treatments ($p < 0.05$).

Parameter	***Chlorella* sp.**		***Desmodesmus* sp.**		***Ankistrodesmus* sp.**	
	T1	**T2**	**T1**	**T2**	**T1**	**T2**
k	0.25±0.01a	0.19±0.01a	0.34±0.00a	0.27±0.00 a	0.26±0.00a	0.23±0.00a
dt	2.81±0.16a	3.74±0.15a	2.06±0.00a	2.57±0.01 b	2.65±0.00a	3.00±0.00a
y (cell/mL x106)	1.0±0.014b	1.2±0.091a	1.1±0.045b	1.3±0.020 a	2.15±0.65b	2.18±0.48a
md (cell/mL x106)	8.0±0.16b	10.8±0.66a	7.8±0.034b	15.6±0.21 a	23.1±0.19b	27.9±0.37a

Post-thawing cell viability

Chlorella sp. at day 0: The highest percentage of NCD cells were recorded in T3 (20.36 ± 1.27 %) and T4 (22.30 ± 1.27 %) and the lowest in T1 (1.87 ± 0.46 %), T2 (1,19 ± 1.16 %), and T5 (0.30 ± 0.25 %; $p < 0.05$), forming three groups (T1-T2-T5; T3-T4; and T6). The lowest CD was observed in T2 (8.91 ± 1.45 %) and the highest in T6 (41.12 ± 0.57 %, $p < 0.05$), consolidating four groups (T1-T3, T2, T4-T5, and T6). The lowest ML was found in T4 (41.41 ± 0.57 %) and T6 (43.43 ± 1.14 %) differing from the other treatments ($p < 0.05$), generating five groups (T1, T2, T3, T4-T6, and T5; Figure 2a).

Chlorella sp. at day 5: The highest NCD was recorded in T6 (24.6 ± 1.08 %) and the lowest in T5 (3.6 ± 0.38 %) and T2 (4.6 ± 0.51 %; $p < 0.05$), forming four groups (T1, T2-T5, T3-T4, and T6). The lowest CD was found in T1 and T5 (38.2 ± 0.43 and 39.78 ± 0.96 %, respectively) and the highest in T2 (52.9 ± 1.28

%; p <0.05), forming four groups (T1-T5, T2, T3-T4, and T6). The lowest ML was found in T6 (30.5 ± 0.67 %) and the highest in T5 (56.6 ± 1.24 %; p <0.05), with five groups (T1; T2; T3-T4; T5; and T6; Figure 2b).

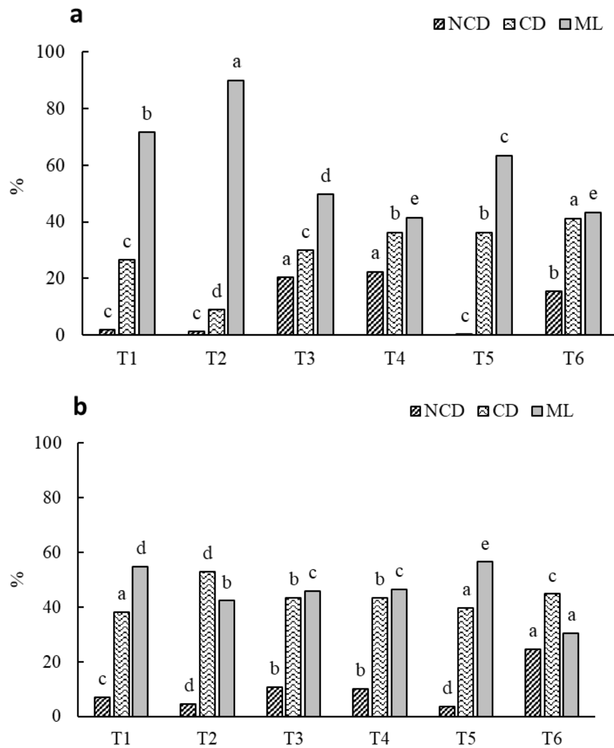


Figure 2. Post-thawing cell viability (PTV) of *Chlorella* sp. a) PTV (%) at day 0. b) PTV (%) at day 5. T1: F/2-5 %, T2: F/2-10 %, T3: NUT-5 %, T4: NUT-10 %, T5: F/2- WOC, T6: NUT- WOC.

Desmodesmus sp. at day 0: The highest NCD was recorded in T6 (39.78 ± 3.71 %) and the lowest in T1 (7.22 ± 2.48 %), T2 (8.11 ± 2.95 %), T4 (4.38 ± 4.26 %), and T5 (4.00 ± 3.25 %; p <0.05), forming three groups (T1-T2-T4-T5; T3; and T6). The lowest CD was found in T6 (44.59 ± 3.80 %) and the highest in T1 (81.84 ± 6.76 %) and T5 (80.67 ± 4.24 %; p <0.05), with four groups overlapping between treatments and a greater difference in T6. The lowest ML was found in T1 (10.94 ± 7.36 %) and the highest in T4 (27.15 ± 3.96 %), resulting in two groups overlapping between treatments, with a difference between T1 and T4 (Figure 3a).

Desmodesmus sp. at day 5: The highest NCD was recorded in T6 (7.51 ± 1.52 %) and the lowest in T2, T3, T4 and T5 (0.00 ± 0.00 %; p <0.05), forming three groups (T1, T2-T3-T4-T5, and T6). The lowest CD was found in T5 (15.41 ± 5.30 %) and the highest in T6 (74.67 ± 3.17 %; p <0.05), forming four groups without difference between treatments. The lowest ML was found in T6 (17.82 ± 3.26 %) and the highest in T5 (84.59 ± 5.30 %) showing a significant difference (p <0.05), resulting in four groups overlapping between treatments, and differing from T6 (Figure 3b).

Ankistrodesmus sp. at day 0: The highest NCD was recorded in T3 (12.13 ± 0.66 %) and the lowest in T2 and T5 (4.88 ± 0.39, and 4.98 ± 0.50 %, respectively; p <0.05), forming four groups (T1-T4, T2-T5, T3, and T6). The lowest CD was found in T5 (23.39 ± 0.33 %) and the highest in T3 and T4 (62.35 ± 1.29 and 61.47 ± 0.25 %, respectively; p <0.05), with four groups (T1-T2, T3-T4, T5, and T6). The lowest ML was found in T3 (25.52 ± 0.97 %) and the highest in T5 (71.63 ± 0.64 %; p <0.05; Figure 4a).

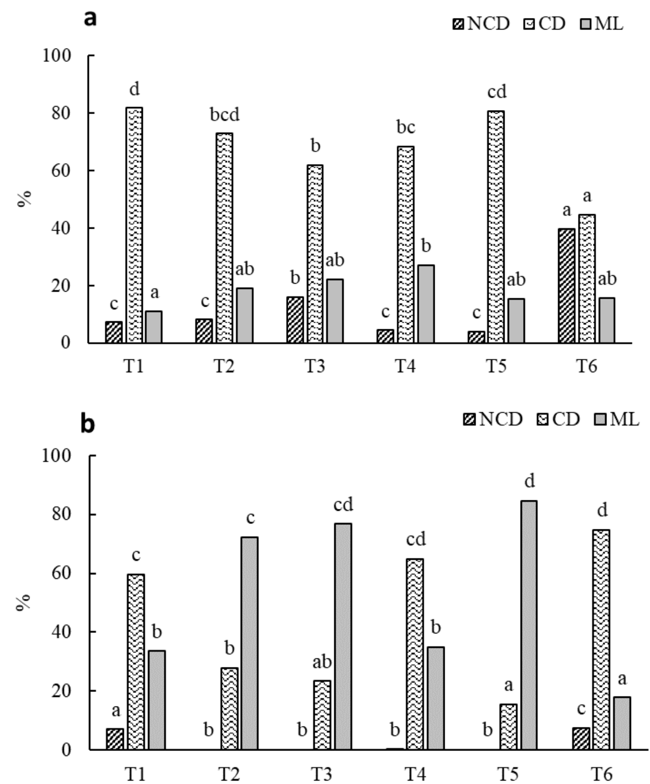


Figure 3. Post-thawing cell viability (PTV) of *Desmodesmus* sp. a) PTV (%) at day 0. b) PTV (%) at day 5. T1: F/2-5 %, T2: F/2-10 %, T3: NUT-5 %, T4: NUT-10 %, T5: F/2- WOC, T6: NUT- WOC.

Ankistrodesmus sp. at day 5: The highest NCD was found in T3 (18.16 ± 0.22 %) and the lowest in T5 (2.97 ± 0.24 %; p <0.05). The lowest CD was found in T5 (57.00 ± 0.61 %) and the highest in T1 and T2 (77.04 ± 1.05 and 76.68 ± 0.87 %, respectively; p <0.05), forming four groups (T1-T2, T3-T4, T5, and T6). The lowest ML was found in T3 (12.10 ± 1.04 %) and the highest in T5 (40.03 ± 0.85 %; p <0.05) making five groups with an overlap between T1 and T2 with T4, and three different

groups (T3, T5 and T6; Figure 4b). As a general trend for the three microalgae, T6 exhibited greater differences.

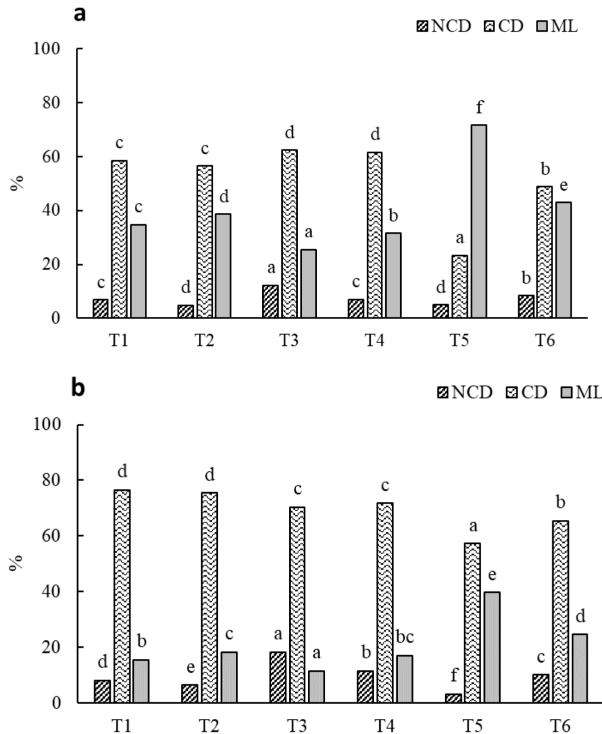


Figure 4. Post-thawing cell viability (PTV, %) of *Ankistrodesmus* sp. a) PTV (%) at day 0. b) PTV (%) at day 5. T1: F/2-5 %, T2: F/2-10 %, T3: NUT-5 %, T4: NUT-10 %, T5: F/2- WOC, T6: NUT- WOC.

Discussion

Population growth

We evaluated the incidence of culture medium on population growth and productive variables of three microalgae species. All species showed a higher instantaneous growth rate and shorter doubling time when F/2 was used. On the other hand, higher yield and cell density was observed when NUT was used. Additionally, each species responded differently to availability and proportion of N and P (Jad, 2012; Prieto, 2013). According to Silva (2016), adequate proportions of urea, ammonium, potassium, phosphorus, magnesium, sulfur, micronutrients and vitamins should be used in foliar fertilizers for *Chlorella sorokiniana* to obtain good growth, high productivity, adequate concentration of chlorophyll, proteins and carbohydrates.

Nitrogen plays a key role in microalgal growth (Tagliaferro *et al.,* 2019) since it is an important part of its biomass (about 5 % in dry matter), particularly of lipids and proteins (Ortega and Reyes, 2012; Hernández and Labbé, 2014; López *et al.,* 2015; Cobos *et al.,* 2016). A N deficiency in the culture medium increases the concentration of reactive oxygen species that can cause damage to the DNA, telomeres and membrane lipids, as well as proteins and carbohydrates in organelles (Ríos, 2003; Benson and Bremner, 2004; Fujita, 2006; Jeyapalan and Sedivy, 2008). Consequently, cells generate protection mechanisms against the effects of free radicals through the action of antioxidants (Bumbak *et al.,* 2011).

Phosphorus and Mg are also vital elements for growth. Phosphorus is involved in energy transfer processes (photosynthesis, nutrient transport, genetic transfer, among others) (Hernández and Labbé, 2014), while Mg is a basic component of chlorophyll, stimulating production of photosynthetic pigments, such as carotenoids, which capture and catalyze the energy of light not absorbed by chlorophyll (Baroli and Niyogi, 2000; Meléndez *et al.,* 2007; Qin *et al.,* 2008). In addition, due to their antioxidant properties, carotenoids protect against oxidative processes (Lazar, 2003; Rodríguez *et al.,* 2010). Additionally, Mg helps to maintain the osmotic pressure and the ionic balance of the cell (Silva, 2016).
The concentration of N, K, P and Mg was higher with NUT, which explains its efficiency in obtaining high microalgal densities. Cobos *et al.* (2016) compared growth of *Chlorella* sp., *Ankistrodesmus* sp. and *Scenedesmus* sp. in CHU10 medium with and without N, finding better growth when the culture medium contained nitrogen. Nitrogen concentration in the medium also influences the nutritional quality of microalgae; proteins and carbohydrates increase with N, and higher lipid content is obtained when N decreases (Cobos *et al.,* 2016; Silva *et al.,* 2016; Bhattacharya and Goswami, 2020).

Chlorella sp. reached a higher density (10.8 ± 0.66 x 106 cells. mL^{-1}) when grown with NUT compared to F/2 medium. A similar trend is observed in other works when using a traditional medium versus an alternative one. In context, Colorado *et al.* (2013) obtained lower densities (2.41x106 cells. mL^{-1}) in 1,200 liters of raceway system culture with Bristol conventional medium. They reported 0.19 and 0.22 instantaneous growth rate in 400 mL and 16 L, respectively, results lower than those observed in the present study using F/2 (in 500 mL). Vera *et al.* (2002), in discontinuous cultures (in 300 mL) with *Chlorella* sp. in Algal medium obtained higher K (0.66), lower dt (1.04) and higher md (32.73 ± 1.09x106 cells. mL^{-1}) than those observed in the present study. In contrast, similar results to this work were reported by Muñoz *et al.* (2012), who obtained 10.9 ± 1.6x106 cell. mL^{-1} in 2.5 liters

using an NPK fertilizer. Similarly, Cobos *et al.* (2016) obtained a lower concentration (2.8 x 106 cells. mL^{-1}) culturing *Chlorella* sp. with an alternative N source. This difference could be due to the fact that different *Chlorella* species respond differently to the culture medium and environmental conditions (Colorado *et al.,* 2013; Cobos *et al.,* 2016).

Few studies have been reported using *Desmodesmus.* Considering that the concentration of Nutrifoliar used was N of 2.0x105 ppm, P of 1.0x105 ppm and K of 5x104 ppm, our results are lower than those of Ortega and Reyes (2012), who compared growth rates and yield of several freshwater species, including *Scenedesmus quadricauda*, in F/2 medium and in two low-cost media based on agricultural fertilizers Fert I (N: 2.4x105 ppm; P: 1.7x105 ppm and K: 1.3x105 ppm) and Fert II (N: 2x105 ppm; P: 3x105 ppm and K: 1x105 ppm). *S. quadricauda* showed a constant increase in cell number (up to a maximum of 157.8 x 106 cells. mL^{-1} in 32 L) with 0.72 k for F/2 medium and 0.64 for Fert II. The results of the present study are greater than those reported by Cobos *et al.* (2014), who cultivated *Scenedesmus* sp. reaching a density of 4 x 106 cells. mL^{-1} in CHU10 medium. Likewise, in a later study, Cobos *et al.* (2016) cultivated *S. quadricauda*, obtaining 13.6 x 106 cells. mL^{-1} and 0.42 k.

The growth and population parameters observed in *Ankistrodesmus* sp. are similar to those reported by Sipaúba and Pereira (2008) who evaluated *Ankistrodesmus gracilis* using NPK (20 % N, 5 % P, 20 % K) as an alternative medium, reporting greater duplication time (4.54 ± 0.41) and lower growth rate (0.22 ± 0.02) than those obtained in the present study. According to Cobos *et al.* (2016), *Ankistrodesmus* sp. reached a density of 15.7 x 106 cells. mL^{-1}, which is lower than that of the present work, and a higher instantaneous growth rate (0.77) in *Akistrodesmus nannoselene* cultivated in CHU10 medium with the addition of nitrogen. Mansa *et al.* (2018), using Bold Basal Medium (BBM) for *Ankistrodesmus* sp., obtained a maximum density of 9.77 ± 0.59 x 106 cells. mL^{-1}, a higher instantaneous rate of specific growth (0.43 ± 0.04), and a shorter doubling time (1.63 ± 0.15 days). Prieto (2013), evaluating *Ankistrodesmus* sp. in 250 mL in Conway and F/2 media, achieved densities of 12.6 ± 0.16 and 18.4 ± 0.28 x 106 cells. mL^{-1}, with growth rates of 0.10 and 0.12 for each treatment, respectively. These values are lower than those obtained in the present study. From the above it can be inferred that Nutrifoliar fertilizer improves growth and productive parameters of *Ankistrodesmus* sp. and it is an alternative low-cost culture medium that increases yield and crop density of the studied microalgae.

Post-thawing cell viability

Greater post-thawing cell viability (PTV) was observed when microalgae were previously cultured with Nutrifoliar (NUT). This is due to the fact that foliar fertilizers generally have in their composition molecules that are easily assimilated by the plant and can act as cryoprotectants by making the osmotic environment more pleasant, making the freezing of the microalgae gradual and reducing the formation of ice crystals that compromise the integrity of the organelles, in addition to the support of microalgal growth (Holm-Hansen, 1965; Colinagro, 2013; Silva, 2016). Vásquez *et al.* (2013) point out that the culture medium and the environmental conditions determine the physiological state and biochemical composition of microalgal cells.

According to the above, adequate concentrations of assimilable nutrients in the culture medium stimulate cellular protection mechanisms (structural and functional) against variations in temperature, light, centrifugation, freezing and thawing of microalgae. The adequate concentration of nutrients can favor the physiological processes of the cell to counteract the damaging effects of cryopreservation, allowing its optimization through the formation of aldose type monosaccharides, which increases the osmolarity of the solution, causing cellular dehydration. These aldoses in synergy with cryoprotectors, minimize the damage of intracellular ice by increasing the total concentration of the solute and reducing the amount of ice formed in the cell (Bui *et al.,* 2013; Aray-Andrade *et al.,* 2018).

The cryoprotectant is essential for a successful microalgal cryopreservation process. The selected agent must have low molecular weight to easily penetrate the cell membrane (Castañeda *et al.,* 2010; Ji, *et al.,* 2013; Hazen, 2013), be highly soluble in water, have low toxicity, low reactivity, and not precipitate at a high concentration (Prakash *et al.,* 2012). It must protect the cell from injuries caused by the formation of ice crystals, which induce physical and chemical changes (Mazur, 2004; Wowk, 2007). Permeable cryoprotectants are ideal, since they reduce the intracellular-water freezing point, formation of hydrogen bonds, vitrification of solvents, and prevent the formation of ice crystals inside the cell (Fuller, 2004; Chian, 2010); all factors that can cause irreversible cell damage (Day and Fleck, 2015).

Methanol (MET) is a commonly used cryoprotectant since it meets these characteristics (Jain and Paulson, 2006) and has proven its effectiveness. Abreu *et al.* (2012) evaluated cryopreservation of *Thalassiosira weissflogii*, *Nannochloropsis oculate* and *Skeletonema* sp with slow freezing or direct immersion in liquid N using DMSO or MET as cryoprotectants. They reported better post-thawing viability for *T. weissflogii* microalgae using 10 % DMSO and 5 % MET. *N. oculata* showed good results with and without both cryoprotectants; whereas, *Skeletonema* sp. did not have viability under these conditions. These results are similar to those in the present study where less cellular damage was observed with 5 % and 10 % methanol, and in some cases, there was a positive response even when cryoprotectant was not added. Thus, there is a relationship between the cryoprotectant used and the microalgae species; therefore, the cryoprotectant and its concentration must be specific for each species.

Regarding cell viability, it depends on the microalgae and the protocol used (Avila and Llanos, 2014). Hwanc and Horneland (1965) used a controlled freezing rate up to -30 °C after inclusion in liquid N, obtaining 100 % viability for several strains of *Chlorella* sp. Prieto *et al.* (2017) evaluated *Ankistrodesmus* sp., finding that 5 % methanol resulted in the highest percentage of cells without damage (79.3 % ± 2.82 %), the lowest with cellular damage (15.04 % ± 0.95 %), and the lowest with marked lesions (5.68 % ± 0.18 %).

Nutrifoliar complete fertilizer (0.99 mL /500 mL) can be used as an alternative low-cost culture medium for producing freshwater phytoplankton from microalgae *Chlorella* sp., *Desmodesmus* sp. and *Ankistrodesmus* sp. This fertilizer allows the obtention of high density yield, and adequate population parameters in short cultivation periods, as well as greater cellular viability after cryopreservation. Advanced techniques should be used to more accurately identify the structural and molecular damages that occur in microalgae.

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