

Cellular feasibility study of *Acutodesmus obliquus*

Estudo de viabilidade celular do *Acutodesmus obliquus*

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Abstract

The development and validation of efficient microalgae conservation methods is essential for the establishment and constitution of long-term culture collections, as well as for programming for breeding and possible genetic modification of algae. However, each species of microalgae demonstrates responses considered unpredictable, and thus, making it difficult to standardize universal methods so far. The results presented in this study indicate time as a limiting factor for the conservation of *A. obliquus*, in the methods of conservation by refrigeration, freezing and freezing with the use of cryoprotectants, such as DMSO, Gli and Poly. Freezing with the use of cryoprotective substances demonstrated satisfactory efficiency in the conservation of microalgae, and thus, surprisingly, in conservation by refrigeration. As already known, conservation by freezing only showed low efficiency, since the cells are broken by the formation of ice crystals in their interior, and thus, making the microalgae conservation unfeasible for a longer period. The study effectively showed the conservation of *A. obliquus* by refrigeration and freezing with the use of cryoprotective substances mentioned in this work.

Keywords: *Acutodesmus obliquus*; Cell viability; Freezing; Cooling; Cryoprotective substances.

Resumo

O desenvolvimento e validação de métodos eficientes de conservação de microalgas é essencial para o estabelecimento e constituição de coleções de culturas de longo prazo, assim como também na programação para melhoramento e possível modificação genética de algas. Entretanto, cada espécie de microalga demonstra repostas consideradas imprevisíveis, e assim, dificultando a padronização de métodos universais até o momento. Os resultados apresentados neste estudo indicam o tempo como fator limitante para conservação do *A. obliquus*, nos métodos de conservação por refrigeração, congelamento e congelamento com uso de crioprotetores, como DMSO, Gli e Poli. O congelamento com uso de substâncias crioprotetoras demonstraram satisfatória eficiência na conservação da microalga, e assim, como também de forma surpreendente na conservação por refrigeração. Como já sabido, a conservação por somente congelamento demonstrou baixa eficiência, uma vez que é causada a ruptura das células pela formação de cristais de gelo em seu interior, e assim, inviabilizando a conservação da microalga de forma mais prolongada. O estudo evidenciou com eficácia a conservação do *A. obliquus* por refrigeração e por congelamento com o uso de substâncias crioprotetoras citadas neste trabalho.

Palavras-chave: *Acutodesmus obliquus*; Viabilidade celular; Congelamento; Resfriamento; Substâncias crioprotetoras.

Resumen

El desarrollo y validación de métodos eficientes de conservación de microalgas es esencial para el establecimiento y constitución de colecciones de cultivos a largo plazo, así como para la programación de reproducción y posible modificación genética de algas. Sin embargo, cada especie de microalga muestra respuestas consideradas impredecibles y, por lo tanto, dificulta la estandarización de los métodos universales hasta el momento. Los resultados presentados en este estudio indican que el tiempo es un factor limitante para la conservación de *A. obliquus*, en los métodos de conservación por refrigeración, congelación y congelación con el uso de crioprotectores, como DMSO, Gli y Poly. La congelación con el uso de sustancias crioprotectoras demostró una eficacia satisfactoria en la conservación de microalgas y, por tanto, sorprendentemente, en la conservación por refrigeración. Como ya se sabe, la

conservación por congelación solo mostró una baja eficiencia, ya que las células se rompen por la formación de cristales de hielo en su interior y, por tanto, hacen inviable la conservación de las microalgas por un período más largo. El estudio mostró efectivamente la conservación de *A. obliquus* mediante refrigeración y congelación con el uso de sustancias crioprotectoras mencionadas en este trabajo.

Palabras clave: *Acutodesmus obliquus*; Viabilidad celular; Congelación; Enfriamiento; Sustancias crioprotectoras.

1. Introduction

Microalgae are very diverse beings present in aquatic systems, they are considered photoautotrophic beings, that is, they have the ability to synthesize their own nutrients with high photosynthetic efficiency, which are constituted by important sources of lipids, hydrocarbons and other oils, destined for production of biofuels (Choi et al. 2019; Preisig e Andersen, 2005). In addition, microalgae make use of nutrients, carbon dioxide and photosynthetic agents to grow and multiply (Selvan et al. 2019; West, 2005). In view of this, microalgae are classified into large groups and diversity of structural characteristics, being able to reproduce and survive in adverse conditions (Branco et al. 2020).

Among the existing microalgae, *Acutodesmus obliquus* (*A. obliquus*) stands out, also known as *Tetradesmus obliquus*, as classified in the Algaebase and defined by Hegewald and Hanagata (2000). The cell wall of *A. obliquus* is made up of two main layers, the inner layer being mainly composed of polysaccharides such as cellulose, and the outer layer being represented by a trilaminar structure composed of algaenan, a highly resistant biopolymer that acts as a barrier to recovery of intracellular components (Choi et al. 2019; Wynne e Hallan, 2015).

According to D'Alessandro (2018), the cellular constitution of *A. obliquus*, the microalgae is capable of producing fatty acids ranging from C14:0 to C22:0, with C16:0 and C18:1 being the majority, as also observed in other works such as Aslani et al. (2018) and Soares et al. (2014), being considered a biomass with great potential in the production of biofuels, as verified in the studies by Selvan et al. (2019), with the production of 98.74% of biodiesel and 96.83% of bioethanol. In addition to the production of biofuels using different microalgae biomasses, there are several studies also applied to microalgae in the production of pigments, such as lutein, astaxanthin, phycobilins, chlorophylls and carotenoids, with high commercial value and potential economic support in the production of biodiesel (D'Alessandro et al. 2019; D'Alessandro e Antoniosi Filho, 2016).

Microalgae biomass can be effectively preserved through storage, however, there are few studies that show cell viability techniques when the biomass is refrigerated, or even frozen for long periods of time. The freezing method becomes unfeasible mainly due to the formation of ice crystals, being responsible for cell expansion, causing cell wall disruption (Hanashiro et al. 2019; Kapoore et al. 2019; Tessarolli, Day, Vieira 2017; Umamaheswari e Shanthakumar, 2016).

Therefore, the objective of this study is to evaluate the cell viability of *A. obliquus* under different storage conditions, such as cooling, freezing and freezing added with different cryoprotective agents, and thus, knowing the storage time limit with permanence of viable cells in medium of cultivation.

2. Methodology

The experiment was carried out at the Laboratory of Environmental Microbiology and Biotechnology at the Federal University of Goiás – UFG – Campus Goiânia. The following work aims to carry out a case study, with the objective of evaluating the conditions inherent to the cell viability of *A. obliquus*, under different storage circumstances, such as cooling, freezing and freezing added with different cryoprotective agents, and thus, aiming to know the maximum storage time with permanence of viable cells, for a possible recultivation of microalgal biomass.

2.1 Preparation of the BBM medium

For the cultivation of *Acutodesmus obliquus*, the liquid culture medium BBM, proposed by Andersen et al. (2005), and presented in Table 1.

2.2 Cell viability study of *Acutodesmus obliquus*

105 test tubes were prepared containing 5mL of BBM medium, being performed in triplicate, and programmed for 6 months under the following storage conditions: 1. refrigerated biomass; 2. frozen biomass + DMSO (dimethylsulfoxide); 3. frozen biomass + Gly (glycerol); 4. frozen biomass + Poly (polyethylene glycol); and 5. frozen biomass.

In each test tube, *A. obliquus* was inoculated, and placed for growth in an incubator under 16 W white fluorescent lamp lighting, with a photoperiod of 24 hours, and a temperature of 29 °C, for a period of 21 days. Subsequently, the microalgae biomass was concentrated, removing the supernatant. For preservation with the use of cryoprotectants, the biomass was transferred to eppendorf and added with cryoprotective solutions DMSO, Gli and Poly, at a concentration of 10%, and occupying a maximum volume of 1.5 mL, while under refrigeration conditions only and freezing, only the BBM medium was added, and then being stored. The samples that will be frozen will be stored at a temperature of -18 °C, approximately, whereas the samples that will be refrigerated will be stored at a temperature of 4 °C, approximately. Samples were taken in triplicate at time periods zero, 30, 60, 90, 120, 150 and 180 days to conduct the cell viability study.

The cell viability study was conducted using a Neubauer chamber to perform the count of viable and non-viable cells. Beforehand, samples were removed and conditioned at room temperature, and then the sample was centrifuged at 10,000 RCF for 1 minute. Then, the supernatant solution was removed and 1.5 mL of BBM medium was added. Samples were vortexed and shaken at 1500 rpm for 1 minute. Soon after, 10 µL of 10% neutral red dye was added to each sample, and left to rest for 30 minutes in a dark room. With the cover slip placed in the Neubauer chamber, 500 µL of the medium containing the microalgae biomass with neutral red dye were collected and transferred over the edge of the cover slip, carefully filling the counting chamber, and waiting for 2 minutes for sedimentation.

The counting of viable and non-viable cells was performed by observing the four areas of the counting chamber using a fluorescent microscope. The calculation to obtain the number of cells mL⁻¹ was performed using the following equation:

$$\text{number of cells mL}^{-1} = \frac{N1}{N2} \times fd \times 10.000 \quad \text{Equation 1}$$

N1 corresponds to the total number of cells counted in the four areas of the counting chamber, while N2 checks the number of counted quadrants. The fd represents the dilution factor, which in this case is equal to 1, because it was not necessary to do the dilution.

2.3 Calculating Percentage of Viable Cells

The calculation of the percentage of viable cells was determined by applying the following formula:

$$\% \text{ Viable Cells} = \frac{(CtMt - CvMv)}{CtMt} \times 100 \quad \text{Equation 2}$$

Where, Ct represents the total concentration of cells in the original cell suspension, Mt is the multiplication factor used for the total cell count (dilution factor for reading under a microscope = 10), Cv indicates the concentration of non-viable cells, and the Mv is the multiplication factor used for counting non-viable cells (dilution factor for reading under a microscope = 10).

2.4 Cell unfeasibility speed

The calculation to determine the development speed of non-viable cell formation of the microalgae *A. obliquus* was performed after applying the polynomial trend line, and obtaining the speed equation and the coefficient of determination.

Table 1: Composition of BBM medium.

Components	Stock solution (g.L ⁻¹ dH ₂ O)	Quantity used (mL)	Middle concentration at the end
Macronutrients			
NaNO ₃	25,00	10	2,94 x 10 ⁻³
CaCl ₂ .2H ₂ O	2,50	10	1,70 x 10 ⁻⁴
MgSO ₄ .7H ₂ O	7,50	10	3,94 x 10 ⁻⁴
K ₂ HPO ₄	7,50	10	4,31 x 10 ⁻⁴
KH ₂ PO ₄	17,50	10	1,29 x 10 ⁻³
NaCl	2,50	10	4,28 x 10 ⁻⁴
Alkaline EDTA Solution		1	
EDTA	50,00		1,71 x 10 ⁻⁴
KOH	31,00		5,53 x 10 ⁻⁴
Acidified Iron Solution		1	
FeSO ₄ .7H ₂ O	4,98		1,79 x 10 ⁻⁵
H ₂ SO ₄		1	
Boron Solution		1	
H ₃ BO ₃	11,42		1,85 x 10 ⁻⁴
Trace Metals Solution		1	
ZnSO ₄ .7H ₂ O	8,82		3,07 x 10 ⁻⁵
MnCl ₂ .4H ₂ O	1,44		7,28 x 10 ⁻⁶
MoO ₃	0,71		4,93 x 10 ⁻⁶
CuSO ₄ .5H ₂ O	1,57		6,29 x 10 ⁻⁶
Co(NO ₃) ₂ .6H ₂ O	0,49		1,68 x 10 ⁻⁶

Fonte: Andersen, et al (2005).

3. Results and Discussion

According to Lourenço (2006), concentrated microalgae biomass does not necessarily need to be used immediately after its collection, but can be preserved for some time before its use, through procedures such as refrigeration and freezing with the use of cryoprotective substances.

Table 2: Quantitative percentage of non-viable cells by period of time in days for each type of storage:

Cell condition	Percentage of non-viable cells/day \pm sd						
	0	30	60	90	120	150	180
Chilled biomass	0,00	47,93 \pm 0,34	63,35 \pm 0,45	73,98 \pm 0,27	85,16 \pm 0,18	93,02 \pm 0,29	98,63 \pm 0,12
Frozen biomass + DMSO	0,00	38,94 \pm 0,72	57,04 \pm 0,51	70,51 \pm 0,56	84,97 \pm 0,39	93,50 \pm 0,32	97,01 \pm 0,26
Frozen biomass + Gli	0,00	44,86 \pm 0, 31	61,78 \pm 0,21	69,61 \pm 0,38	88,54 \pm 0,25	95,35 \pm 0,21	97,79 \pm 0,18
Frozen biomass + Poly	0,00	41,47 \pm 0,24	59,98 \pm 0,47	71,44 \pm 0,42	89,45 \pm 0,41	94,05 \pm 0,19	98,75 \pm 0,24
Frozen biomass	0,00	57,25 \pm 0,19	71,19 \pm 0,32	92,81 \pm 0,29	100,00 \pm 0,00	100,00 \pm 0,00	100,00 \pm 0,00

sd: standard deviation. Fonte: Autores.

In this study, neutral red was used, which is a cationic dye considered weak and soluble in water, acting as a marker, in this case in the identification of live *A. obliquus* cells. This dye permeates the plasma membrane of the cell, which is intact and is concentrated exactly in the cytoplasm and/or vacuoles of viable microalgae cells, turning it red. However, the cells of the microalgae that somehow suffered damage, that is, they are not viable, during the conservation methods, the incorporation of the dye by the cytoplasmic membrane is inhibited, and the microalgae is declared with cellular inviability (Da Luz et al., 2016).

In the following study, conservation by simple freezing revealed low effectiveness of cell viability, which showed a significant decrease in viable cells, reaching a minimum amount of live cells in a maximum period of 90 days of conservation, with the number of cells in 90 days expressively minimal viable cells, with a maximum of 7.19% of viable cells, as shown in Table 2. According to Da Luz et al. (2016), in the case of conservation by simple freezing, this technique involves the death of all or almost all cells in a short period of time, and entails changes, whether small or null, in the chemical characteristics of the algal material.

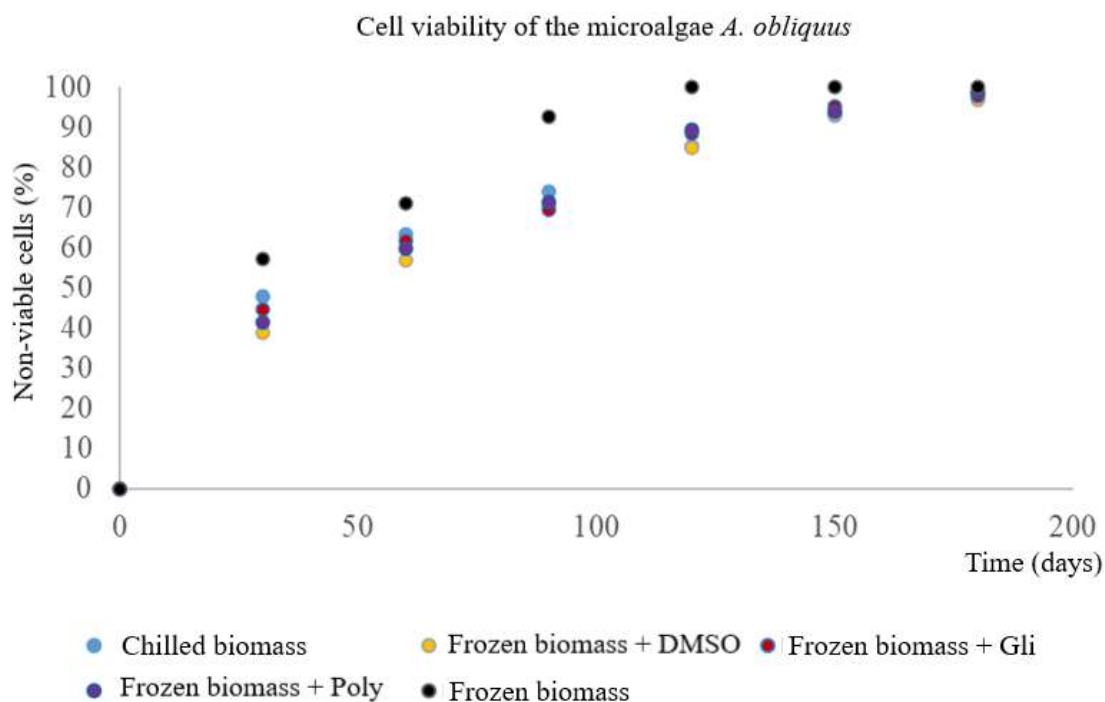
The *A. obliquus* that was stored under refrigeration conditions, frozen + DMSO, frozen + Gli, frozen + Poly, and frozen, showed a marked reduction in the number of viable cells in the medium which were preserved for a maximum period of 180 days, as shown in Table 2. Only in the first thirty days was there a marked reduction of 47, 93%, 38.94%, 44.86%, 41.47% and 57.25% of viable cells in the samples, respectively, and occurring progressively in the remaining 150 days of storage, being less impactful than in the first 30 days.

According to Da Luz et al. (2016), rarely in the freezing method is microalgae culture used in a liquid medium, and the cultures are previously concentrated, if there is a large amount of water between the cells, it will cause large losses of viable cells, and this is due to the rupture of the cells by the formation of ice crystals inside. However, the use of cryoprotecting substances contributes to the promotion of cell viability, acting both extracellularly and intracellularly, for example, as in the case of glycoprotective substances that are permeable agents, such as dimethylsulfoxide (DMSO) and glycerol (Gli), has the function of interrupting the formation of intracellular ice crystals, while polyethylene glycol (Poly), which is a non-permeable agent, induces cell dehydration, promoting intracellular water efflux (Fernandes et al., 2019).

Freezing conservation with the increase of cryoprotective substances such as dimethylsulfoxide, glycerol and polyethylene glycol, used in this study, through the application of strict protocols, contribute to the significant increase in the percentage of living cells during the freezing period, as observed in Table 2, in comparison to conservation by simple freezing. Conservation by simple freezing showed a large drop in the number of live cells, lasting 90 days with the presence of live *A. obliquus* cells, while with the use of cryoprotectants there was a 100% longer survival than by freezing alone, having a maximum duration of 180 days with the presence of live microalgae cells.

As shown in Figure 1, *A. obliquus* frozen in a medium contained with the cryoprotectant dimethylsulfoxide (DMSO), revealed among the cryoprotective substances used in this study to be the best cryoprotective substance as a form of conservation under freezing, with a slower reduction in viable cells, and in consecutive, a lower content of non-viable cells during freezing. The sample that was refrigerated proved to be an alternative, since it showed a low reduction over the 180 days, being similar to the samples that showed the use of cryoprotectants during conservation.

Figure 1: Representation of the percentage of viable cells over time.



Fonte: Autores.

Conservation by cooling proved to be quite effective in relation to the method of conservation by freezing with the use of cryoprotective substances, despite being considered by Lourenço (2006) as a form of conservation with a short shelf-life for algal biomass. The temperature of 4 °C used in this study proved to be sufficient to delay any deterioration processes in the biomass of *A. obliquus*, as shown in Table 2.

Table 3 shows exactly what is seen in Table 2, which shows a significant increase in the rate of non-viability of *A. obliquus* cells in the first thirty days, reaching values 1.60%, 1.30%, 1.50 %, 1.38% and 1.91% for chilled, frozen + DMSO, frozen + Gli, frozen + Poly and frozen biomass, respectively. The cell inviability speed is more evident when the biomass is only frozen, having a significant number of microalgae cell death.

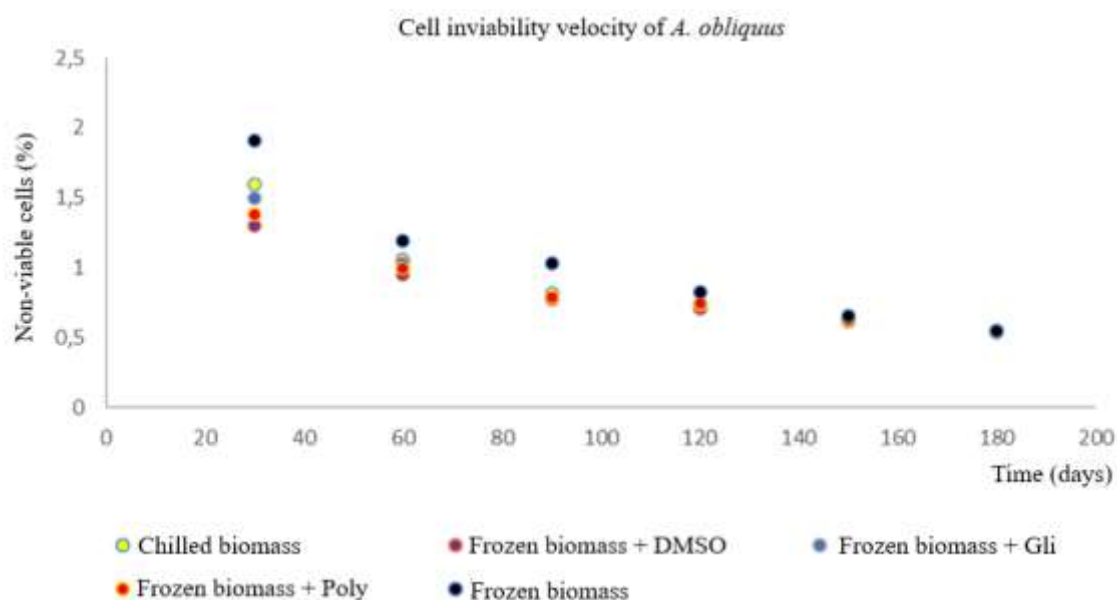
Table 3: Verification of cell unfeasibility speed and cell unfeasibility speed equations by type of storage.

Cell condition	Cell unfeasibility speed (% day ⁻¹)						Cell unfeasibility velocity equation	R ²
	30	60	90	120	150	180		
Chilled biomass	1,60	1,06	0,82	0,71	0,62	0,55	$V_{cnv} = 6E-05t^2 + 0,0187t + 2,053$	0,9767
Frozen biomass + DMSO	1,30	0,95	0,78	0,71	0,62	0,54	$V_{cnv} = 3E-05t^2 + 0,0116t + 1,581$	0,9770
Frozen biomass + Gli	1,50	1,03	0,77	0,74	0,64	0,54	$V_{cnv} = 5E-05t^2 + 0,0161t + 1,885$	0,9622
Frozen biomass + Poly	1,38	1,00	0,79	0,75	0,63	0,55	$V_{cnv} = 4E-05t^2 + 0,0128t + 1,690$	0,9716
Frozen biomass	1,91	1,19	1,03	0,83	0,66	0,55	$V_{cnv} = 6E-05t^2 + 0,0207t + 2,389$	0,9625

Fonte: Autores.

The increasing quantity of non-viable cells shown in Figure 1 is expressive, as well as, in contrast, the decreasing quantity of cell invariability rate of *A. obliquus*, represented by Figure 2, regarding the 180 day conservation period. Both Figure 1 and Figure 2 reveal that in the case of microalgae, time is a limiting factor for conservation by refrigeration or freezing, even with the use of cryoprotectants, being much more evident, especially in the simple freezing method. The use of cryoprotective substances proved to be a very efficient methodology. Conservation by simple cooling with concentrated biomass proved to be very surprising to be a methodology comparable to the methodology of application in freezing with the use of DMSO, Gli and Poly cryoprotectants. It is also observable through Table 3, a very close similarity after 30 days of conservation, the decreasing speed of cellular invariability of the microalgae between the refrigeration and freezing methods with cryoprotectants.

Figure 2: Representation of *A. obliquus* cell unfeasibility rate in relation to time.



Fonte: Autores

As verified, time proved to be a limiting factor for microalgae conservation, as well as temperature, biomass concentration and the use of cryoprotective substances. Table 3 reveals exactly the significant decrease in the first 30 days of the cellular invariability speed of the microalgae, and later, a less progressive decrease in the cellular invariability speed, being more expressive for the samples that were only frozen. Both samples that were refrigerated and frozen together with cryoprotectors showed similarity in the drop in cell invariability rate. Through the cellular unfeasibility velocity values, it was possible to describe the cellular unviability velocity equations of each conservation method due to time as a limiting factor, as

shown in Table 3. As well as obtaining the coefficient of determination (r^2), aiming to assess the degree of adjustment of the microalgae cell unfeasibility velocity equation from the data of the time and cell unviability velocity variables.

4. Conclusion

The results presented in this study indicate that the time factor can also be considered determinant for the life of microalgae, in this case for *A. obliquus*, in addition to temperature, biomass concentration and use of cryoprotective substances for successful conservation. The use of cryoprotective substances really proved to be effective in the preservation of the microalgae, as well as, surprisingly, the refrigeration when adopted strict methodological protocols of procedures for handling and conservation of microalgae biomass. The following study revealed that *A. obliquus* can be conserved relatively for a longer period than by simple freezing when applied to refrigeration or even when freezing with the use of cryoprotective substances such as DMSO, Gli or Poly. Given the great importance of cryoprotective agents, it is of paramount importance that in the future new and more efficient cryoprotective substances are discovered for the preservation of microalgae under freezing.

References

- Andersen, R. A., Berges, F. A., Harrison, P. F., & Watanabe, M. M. (2005). Algal Culturing Techniques: Appendix A. Recipes for freshwater and seawater media. Andersen RA, *Elsevier Academic Press*, 429-538.
- Aslani, A., Mohammadi, M., Gonzalez, M. J. I., Sobczuk, T. M., Nazari, M., & Bakhtiar, A. (2018). Evaluation of the potentials and feasibility of microalgae production in Iran. *Bioresource Technology Reports*, (1): 24-30. <https://doi.org/10.1016/j.biteb.2017.12.001>
- Branco, K. B. Z. F., Balmant, W., Trevisan, E., Taher, D. M., Mariano, A. B., & Arroyo, P. A. (2020). Phenomenological modeling of *Acutodesmus obliquus* microalgae in situ transesterification. *Biochemical Engineering Journal*, (154): 1-37. <https://doi.org/10.1016/j.bej.2019.107434>
- Choi, W. J., Chae, A. N., Song, K. G., Park, J., & Lee, B. C. (2019). Effect of trophic conditions on microalga growth, nutrient removal, algal organic matter, and energy storage products in *Scenedesmus (Acutodesmus) obliquus* KGE-17 cultivation. *Bioprocess and Biosystems Engineering*, (42): 1225-1234. <https://doi.org/10.1007/s00449-019-02120-x>
- Da Luz, D. S., Da Silva, D. G., Souza, M. M., Giroldo, D., & Martins, C. M. G. (2016). Efficiency of neutral red, evans blue and MTT to assess viability of the freshwater microalgae *Desmodesmus communis* and *Pediastrum boryanum*. *Phycological Research*, (64): 56-60. <https://doi.org/10.1111/pre.12114>
- D'Alessandro, E. B., & Antoniosi Filho, N. R. (2016). Concepts and studies on lipid and pigments of microalgae: A review. *Renewable and Sustainable Energy Reviews*, (58): 832-841. <https://doi.org/10.1016/j.rser.2015.12.162>
- D'Alessandro, E. B., Soares, A. T., Pereira, J., & Antoniosi Filho, N. R. (2018). Viability of biodiesel production from a thermophilic microalga in conventional and alternative culture media. *Brazilian Journal of Botany*, (41): 319-327. <https://doi.org/10.1007/s40415-018-0459-7>
- D'Alessandro, E. B., Soares, A. T., D'Alessandro, N. C. O., & Antoniosi Filho, N. R. (2019). Potential use of a thermal water cyanobacterium as raw material to produce biodiesel and pigments. *Bioprocess and Biosystems Engineering*, (42): 2015-2022. <https://doi.org/10.1007/s00449-019-02196-5>
- Fernades, M. S., Calsing, L. C. G., Nascimento, R. C., Santana, H., Morais, P. B., Capdeville, G., & Brasil, B. S. A. F. (2019). Customized cryopreservation protocols for chlorophytes based on cell morphology. *Algal Research*, (38): 1-9. <https://doi.org/10.1016/J.ALGAL.2018.101402>
- Hanashiro, R. H. R., Stoco, C. B., Oliveira, T. V., Lenzi, M. K., Mariano, A. B., & Vieira, R. B. (2019). Experimental validation of hindered settling models and flux theory applied in continuous flow process for harvesting *Acutodesmus obliquus*. *The Canadian Journal of Chemical Engineering*, (97): 1903-1912. <https://doi.org/10.1002/cjce.23467>
- Hegewald, E., & Hanagata, N. (2000). Phylogenetic studies on Scenedesmaceae (Chlorophyta). *Archiv für Hydrobiologie, suppl., Algological Studies*, (100): 29-49. https://doi.org/10.1127/algol_stud/100/2000/29
- Kapooore, R. V., Huete-Ortega, M., Day, J. G., Okurowska, K., Slocombe, S. P., Stanley, M. S., & Vaidyanathan, S (2019). Effects of cryopreservation on viability and functional stability of an industrially relevant alga. *Scientific Reports*, (9): 1-12. <https://doi.org/10.1038/s41598-019-38588-6>
- Lourenço, S. O. *Cultivo de microalgas marinhas – princípios e aplicações*. RiMa, 2006, 606 p.
- Preisig, H. R., & Andersen, R. A. (2005). Algal Culturing Techniques: Historical review of algal culturing techniques. Andersen RA, *Elsevier Academic Press*, 1-12.
- Selvan, S. T., Govindasamy, B., Muthusamy, S., & Ramamurthy, D. (2019). Exploration of green integrated approach for effluent treatment through mass culture and biofuel production from unicellular alga, *Acutodesmus obliquus* RDS01. *International Journal of Phytoremediation*, (21): 1305-1322. <https://doi.org/10.1080/15226514.2019.1633255>

Soares, A. T., da Costa, D. C., Silva, B. F., Lopes, R. G., Derner, R. B., & Antoniosi Filho, N. R. (2014). Comparative analysis of the fatty acid composition of microalgae obtained by different oil extraction methods and direct biomass transesterification. *BioEnergy Research*, (7): 1035-1044. <https://doi.org/10.1007/s12155-014-9446-4>

Tessarolli, L. P., Day, J. G., & Vieira, A. A. H. (2017). Establishment of a cryopreserved bionk for the culture collection of freshwater microalgae (CCMA-UFSCar), São Paulo, Brazil. *Biota Neotropica*, (2): 1-9. <https://doi.org/10.1590/1676-0611-BN-2016-0299>

Umamaheswari, J., & Shanthakumar, S. (2016). Efficacy of microalgae for industrial wastewater treatment: a review on operating conditions, treatment efficiency and biomass productivity. *Reviews in Environmental Science and Bio/Technology*, (15): 265-284. <https://doi.org/10.1007/s11157-016-9397-7>

West, J. A. (2005). Algal Culturing Techniques: Historical review of algal culturing techniques. Andersen RA, *Elsevier Academic Press*, 165-188.

Wynne, M. J., & Hallan, J. K. (2015). Reinstatement of *Tetrademus* G. M. Smith (Sphaeropleales, Chlorophyta). *Feddes Repertorium*, (126): 83-86. <https://doi.org/10.1002/fedr.201500021>