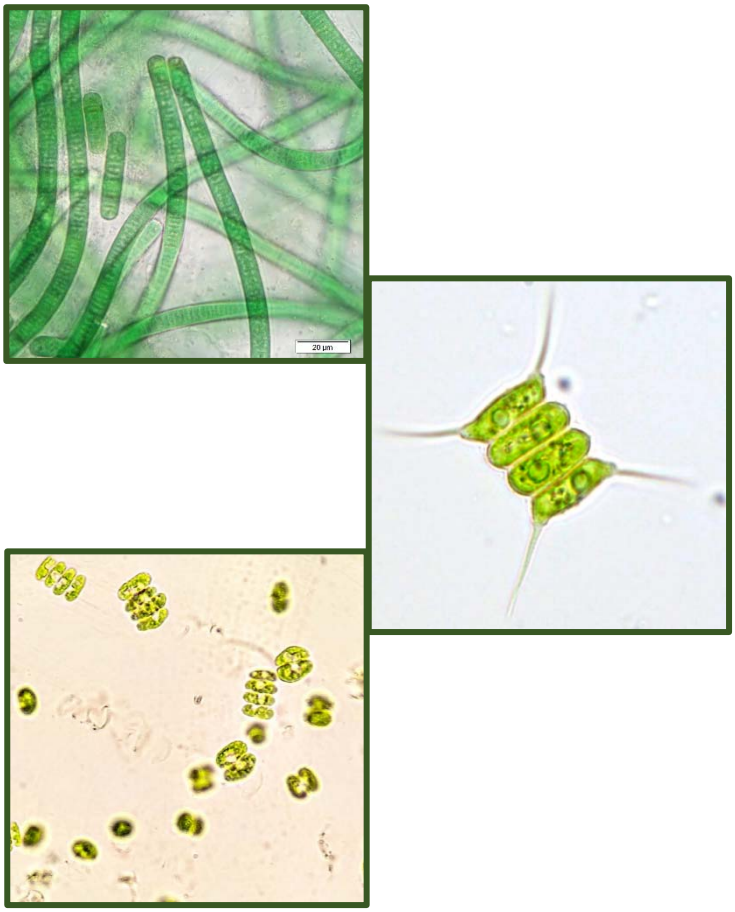
RED CYTED

2024

MICROALGAE PROTOCOLS II



Xavier Álvarez-Montero; Ingrid Mercado-Reyes
EDITORS



RED RENUWAL

MICROALGAE PROTOCOLS BOOK II

(RED CYTED 320RT0005 RENUWAL)

2024

PROGRAMA IBEROAMERICANO

CYTED

CIENCIA Y TECNOLOGÍA PARA EL DESARROLLO

* ISBN: 978-84-15413-62-2

* Editorial: Programa Iberoaméricano de Ciencia y Tecnología para el Desarrollo

* Título: MICROALGAE PROTOCOLS II

* Autor: Álvarez-Montero, Xavier ; Mercado-Reyes, Ingrid

* Formato del producto: Digital: descarga y online
Detalle Formato: PDF

* Idioma de publicación: Español / Castellano

* Número de edición: 1
* Fecha de edición: 12/02/2024
País de edición: España



Web Page	http://www.cyted.org/renuwal
Instagram	https://www.instagram.com/renuwalcyted/
Facebook	https://www.facebook.com/Red-Renuwal-Cyted-108000924395230
YouTube	https://www.youtube.com/channel/UCngLLozh_LLgZP7J_fLUW9A
ResearchGate	https://www.researchgate.net/profile/Renuwal-Cyted-2
LinkedIn	https://www.linkedin.com/in/renuwal-cyted-9081b2205/

INDEX

DETERMINATION OF STEROID CONSUMPTION IN CYANOBACTERIA BY TLC 1

CULTURE OF *Arthrospira platensis* IN MEDIA FORMULATED WITH COMMERCIAL GRADE NITROGEN SOURCES. 8

CULTURE OF MICROALGAE IN DAIRY WASTEWATER: PHYLOGENETIC ANALYSIS AND BIOCHEMICAL COMPOSITION 20

EXTRACTION AND QUANTIFICATION OF MYCOSPORINES FROM *Chlorella vulgaris* FOR SUNSCREEN FORMULATION 38

EXTRACTION AND QUANTIFICATION OF BETA-CAROTENES FROM *Chlorella vulgaris* USING SPECTROPHOTOMETRIC METHOD 41

COLORIMETRIC DETERMINATION OF ANIONIC SURFACTANTS BY MBAS METHOD IN SURFACE WATERS AND WASTEWATER 45

DETERMINATION OF PROTEINS IN MICROALGAL BIOMASS 55

OBTAINING PHOTOSYNTHESIS RATE MODELS AS A FUNCTION OF LIGHT USING PHOTO-RESPIROMETRY 63

THE ANTIOXIDANT POTENTIAL OF MICROALGAL BIOMASS USING THE OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC) METHOD 72

LONG-TERM STORAGE OF PHOTOAUTOTROPHIC MICROORGANISMS ON SOLID CULTURE MEDIUM 80

A SIMPLE AND ALTERNATIVE SCREENING METHOD TO RAPIDLY ASSESS ENZYMATIC HYDROLYSIS OF ALGAL BIOMASS 85

ELECTROFUSION IN CHLORELLA VULGARIS (CHLORELLACEAE) MICROALGUE STRAINS ISOLATED IN COSTA RICA. 95

FOREWORD:

Dear reader, it is a privilege for us to present the compilation of microalgae research protocols II editions, carried out in the Renuwal NETWORK of the Ibero-American Program of Science and Technology for Development (CYTED) during the year 2023. As has been justified, everyday ***microalgae biotechnology*** is becoming more important, with its multiple consolidated applications such as food ingredients, functional foods, feed, nutraceuticals, pharmaceuticals and cosmeceuticals, and emerging applications such as biofuels, biofertilizers, wastewater bioremediation, biofibers, and bioplastics. Microalgae are organisms that can present a prokaryotic (cyanobacteria) or eukaryotic (chlorophytes, euglenophytes, dinophytes, cryptophytes, etc.) organization, with great photosynthetic capacity and high growth rates. However, cyanobacteria are not strictly microalgae, since they are Gram-negative bacteria, and in general terms, microalgae are unicellular or multicellular eukaryotes, but due to their great economic importance they are placed in this context, this added to their plasticity and wide diversity makes these microorganisms an important and sustainable biological resource. The objective of RENUWAL is to promote the use of microalgae for the recovery of nutrients contained in various effluents, thus contributing to improving the sustainability of a multitude of processes. However, to achieve efficient processes, it is necessary to evaluate each application in detail based on the effluents that are going to be processed. Depending on the process, the algal biomass obtained may be of interest for its application in different sectors and final products with different added value, i.e., pharmaceutical, cosmetic, agri-food-livestock-aquaculture, environmental, chemical, or even energetic. Through the RENUWAL Network, we want to create a multi- and interdisciplinary Network that allows the necessary synergy to promote the potential applications of microalgae as recycling agents for industry and the environment within the framework of the Circular Economy. To this end, the available procedures based on microalgae will be analyzed and implemented to allow the development of new sustainable strategies for the valorization of algal biomass. The papers presented focus on protocols used by researchers in the network for the development of their research in the field of microbial biotechnology. The coordinated work of the researchers that make up the network has allowed the publication of a wide spectrum of protocols. We want to congratulate the researchers that make up the network for the great effort made to provide results that are transferred among all countries with the common objective of being able to disseminate information that allows the development of microalgae biotechnology throughout Iberoamerica. The works presented by the network are one more valuable contribution to publicizing the

possibilities of using these important biological resources. We want to thank the CYTED program for providing the opportunity for this interaction between institutions and researchers in the RENUWAL network.

Xavier Álvarez Montero

Ingrid Mercado Reyes

Editors

DETERMINATION OF STEROID CONSUMPTION IN CYANOBACTERIA BY TLC

Govinda Guevara[1], Juana María Navarro Llorens[1]
[1]Grupo de Ingeniería metabólica Departamento de Bioquímica y Biología Molecular, Universidad Complutense de Madrid, 28040 Madrid, España
Correspondence: joana@bio.ucm.es

SUMMARY
This protocol allows for determining the presence of steroid consumption by cyanobacteria in wastewater.

INTRODUCTION
In recent years, the use of cyanobacteria for various purposes has increased, including applications in the food and pharmaceutical industries, agriculture, biofuels and high-value-added products industries, and wastewater treatment (Garlapati et al., 2019; Saeed et al., 2022; Ahmad, 2022). Regarding the latter, wastewater treatment is a necessary process due to the pollution caused by the uncontrolled discharge of domestic and industrial wastewater into the environment. Cyanobacteria may be incorporated into this scenario due to their ability to tolerate high levels of contamination and demonstrate high efficiency in degrading persistent organic pollutants (POPs) and accumulating heavy metals (El-Bestawy, 2008). Furthermore, genetic engineering may improve cyanobacteria's bioremediation capacity against environmental contaminants, allowing them to be used as a low-cost and low-maintenance technology for treating contaminated sites (El-Bestawy, 2008; Zahra et al., 2020).

Steroids, due to their widespread distribution and chemical structure, are particularly recalcitrant to degradation and are found in sewage water. Their high persistence index allows them to be used as reference biomarkers in certain environmental contamination analyses. Steroids are therefore considered emerging contaminants, and steroid hormones, excreted by humans and farmed animals, are a significant source of environmental endocrine disruptors. Bacteria and microalgae have been studied for their ability to metabolize these compounds (Silva, 2012).

As part of the ALGATEC-CM Project (Development of Microalgae for a Circular Economy, P2018/BAA-4532), our group has isolated different cyanobacteria from sewage water. To evaluate the potential of one such cyanobacterium, a *Cyanobium* named A21.1, for bioremediation against environmental pollutants, its ability to biotransform or biodegrade the steroids cholesterol (CHO) and AD was determined using thin layer chromatography (TLC). While this strain was chosen as a proof of concept, the procedure described herein enables the detection of any cyanobacteria AD and CHO consumption by TLC.

MATERIALS

1. Strains, cell culture and preparation

1. Cyanobacteria strains to be tested.
2. The appropriate medium, in this case, sterile BG11 medium (Supplementary Table 1) was used.
3. The steroid to be tested, we used AD and CHO (TCI, >99.0 %)
4. Cold sterile distilled water.
5. Sterile flasks of 250 mL
6. 15 mL sterile disposable tubes.
7. 50 mL sterile centrifuge tubes.
8. Thermostatic orbital shaker with tubes, racks, and bottle adaptors.
9. Bench centrifuge for Falcon tubes.
10. Spectrophotometer.
11. Freezer.
12. Sterile Hood.
13. Ice or a 4 °C chamber.

2. Steroid extraction

1. Chloroform.
2. 50 mL Falcon tubes.
3. Eppendorf tubes of 2 mL and 1.5 mL.
4. Vortex mixer.
5. Dry block heater.
6. Fume hood.
7. Ice.

3. Thin layer chromatography (TLC)

1. Aluminum TLC Silica gel 60 F254 sheets (Merck) were used as stationary phase. This layer can be cut to the desired size with scissors, but taking care not to disturb the surface, it contains inorganic fluorescent indicators for UV detection of colorless compounds at an excitation wavelength of λ254 nm.
2. The mobile phase used to separate AD, ADD, and 9OHAD was a mix of Hexane/ ethyl acetate (10/4, v/v). The correct choice of solvents for the mobile phase is based on the steroid polarity. A good separation is in the range of 0.3-0.7 Rf value. The most common non-polar solvents to mix are hexane (or petroleum ethers/ligroin) and as a polar component ethyl acetate or alcohols (methanol, ethanol). In case a rise in polarity is needed, acetic acid can be used in a very small quantity. Intermediate polarity can be achieved by using acetone.
3. Standard control samples. For this case, AD, 9-OH-AD, and ADD were used. Each one was dissolved in chloroform at 1 μg/μL.
4. Methanol/sulfuric acid (90/10, v/v) was used as visualization reagent.
5. Two 100 mL or a 250 mL beaker covered with a glass lid.
6. Tweezers.

7. UV lamp (λ 254 nm).
8. Hotplate-stirrer.
9. Hair dryer.

PROTOCOL

A scheme of the whole process is depicted in Figure 1.

1. Culture preparation and intermediates production

1.1 In a 250 mL sterile flask with 50 mL of BG11 medium, inoculate the proper cyanobacteria strain to an initial OD_{750nm} of 0.05.

1.2 Growth of the cultures under continuous illumination (10 μmol photon m^{-2} s^{-1}) at 30 °C in a shaker at 220 rpm.

1.3 Label a set of 1.5 mL tubes to collect the culture aliquots.

1.4 Add the 20 mg of the steroid (AD was used) to the cell suspension, mix, and take 500 μL into the first labeled 1.5 mL tube as the starting point of the conversion.

1.5 Take cultures OD measures at different times. For this experiment, it was taken after 10 and 15 days of the steroid addition.

1.6 Incubate the cell suspension in a shaker at 150 rpm and 30 °C and collect 500 μL from the flask at different times (e.g. 7 days, 15 days, 20 days, and 30 days).

1.7 Depending on the speed of the reaction, the time to take aliquots may vary. The times suggested here are a first approach.

1.8 Freeze aliquots at -20 °C.

2. Steroid extraction

2.1 Let the frozen samples melt on ice.

2.2 From now on, work under a fume hood.

2.3 Transfer the samples into a tube with a wide diameter to make the rupture of the ice layer, here a 50 mL Falcon tube was used.

2.4 Add two volumes of chloroform (1 mL).

2.5 Close tightly the tubes and vortex vigorously for 30 seconds.

2.6 Freeze samples at -80 °C for 15 min. and label 2 mL tubes, then carefully disrupt the frozen aqueous phase with a 1 mL pipette tip and transfer the chloroform phase to a labeled 2 mL tube. Avoid any melting of the aqueous phase. The freeze-drying method like this is useful for removing hydrophilic compounds with the aqueous phase (e.g., sugars, proteins).

2.7 Let the chloroform evaporate completely by setting the open lid of 2 mL tubes into a 60°C dry block heater.

2.8 Transfer the tubes containing the dry samples to a rack and close them tightly.

2.9 Keep the samples at room temperature.

3. Thin layer chromatography (TLC) development

3.1 Cut the TLC sheet into pieces 5 cm high by the needed width. Separation

between samples should be at least 5 mm. During this step handle the plates carefully, and avoid disturbing the adsorbent coating by dirt or vibration. TLC sheet must be manipulated with gloves and very sharp scissors. Only the powdered side of the TLC plate should be spotted.

3.2 Prepare a mixture of the adequate mobile phase, in this case, hexane/ ethyl acetate (10/4, v/v), and pour 5 mL into a developing container, a 100 mL or 250 mL beaker, and cover it with a glass lid, swirl it gently and let it still. The depth of the mobile phase must be less than 8 mm.

3.3 With a blunt pencil, draw a parallel horizontal line across the TLC sheet and make a mark every 5 mm. This will be the guideline to load the samples and it will be the reference for calculating Rf values. It should be separated, at least 1 cm from the plate border. Lightly mark under the line the name of each sample to be laid. From now on, manipulate the TLC and place a pair of tweezers.

3.4 Suspend every sample (from step 2.9) in 100 μL of chloroform or a similar volatile solvent (e.g., hexane, ethyl acetate, or methylene chloride). It is important to suspend each sample just before it is laid on the plate, one by one, otherwise, the solvent will evaporate and the steroid concentration will vary from sample to sample.

3.5 Take 5 μL of the suspended sample and carefully and slowly load them onto the designated location on the TLC plate. To avoid creating larger spots, load 1 μL at a time and allow each drop to dry before applying the next 1 μL drop, until all 5 μL have been applied

3.6 Apply 1 μL of every standard control (1 μg/μL on Chloroform) on the same location on the plate, and let the spot dry between consecutive applications.

3.7 With the marked line down carefully place the TLC plate vertically, standing into the developing container. The plate must be leveled into the mobile phase, otherwise, the developing front will be distorted. Be careful that the solvent does not cover the sample lines.

3.8 Close the beaker with a glass lid, and leave it undisturbed. Allow the TLC to develop until the solvent is about half a centimeter below the top of the plate; then remove it from the container. Immediately, mark the solvent front with a pencil.

4. TLC visualization

4.1 Place the plate to completely dry in a fume hood.

4.2 Lay the plate under a λ254 nm UV light. UV-absorbing compounds will be visible; mark them with a pencil.

4.3 Heat up the dried plate by putting it on a 230°C hotplate stirrer

4.4 To visualize other compounds, briefly dip the plate into the visualization reagent and let it drain.

4.5 Using a hair dryer to dry the plate

4.6 Place the TLC plate for a few seconds on the dried plate previously heated in step 4.3. Spots will start to appear, keep heating until you clearly see all the spots. It is important to be cautious to avoid burning the TLC plate.

4.7 Mark with a pencil every new spot and calculate its Rf value.

4.8 Rf is calculated by taking the distance that the solvent reached from the line where the spot was applied (starting point) to the highest point made in step 3.8. Then, measure the distance from the starting point to the center of the spot. Divide the distance traveled by the compound by the distance traveled by the solvent to get the compound Rf value.

$$Rf = \frac{\text{distance traveled by spot}}{\text{distance traveled by solvent}}$$

Once the *Rf* is obtained, compare each value with those of the standards (see Fig. 1). Spots with the same *Rf* as the standards should be also the same color to be sure they are the same compound.

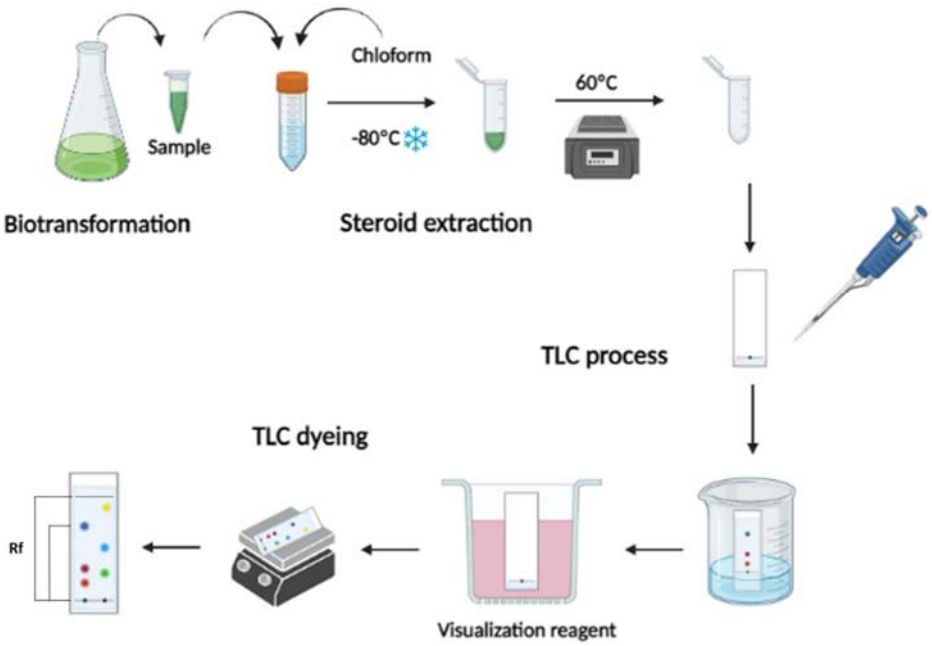


Figure 1. Basic scheme of cell biotransformation and steroid metabolites analysis on an aluminum silica gel TLC plate.

RESULTS

The capability of cyanobacteria to transform steroids was validated for *Cyanobium* A21.1, a strain isolated from sewage water. When cholesterol (CHO) was present in the growth medium, no changes in the steroid were observed over time by TLC (Figure 2). On the other hand, in the case of growth in the presence of AD, a band corresponding to 1,4-androstadien-3,17-dione (ADD) appeared after ten days, suggesting that the cyanobacterium can transform AD into ADD over time (Figure 2).

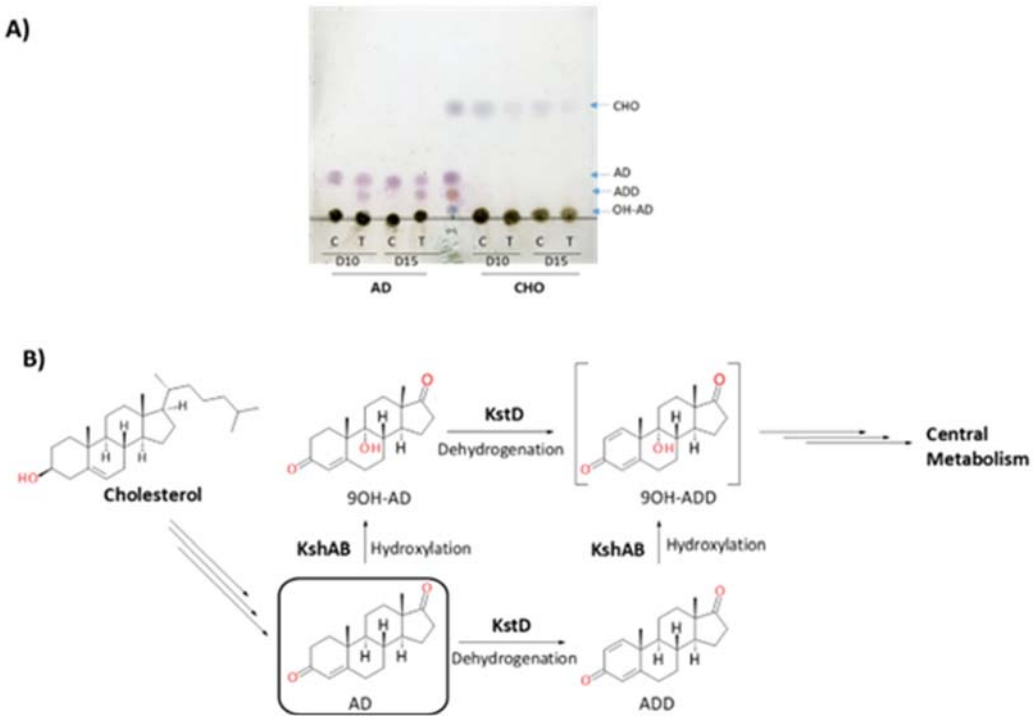


Figure 2. Growth of *Cyanobium* A21.1 on steroids. A) TLC results for growth on AD or CHO. A representative experiment is shown. B) Degradation Pathway of Cholesterol. CHO: cholesterol; AD: 4-androsten-3,17-dione; ADD: 1,4-androstadiene-3,17-dione; 9-OHAD: 9α-hydroxy-1,4-androstadiene-3,17-dione; D: Days; C: control; T: steroid treatment. KstD: 3-Keto-5α-steroid Delta(1)-dehydrogenase; KshAB: 3-Ketosteroid 9α-hydroxylase.

Supplementary Table 1

Table 1. BG11 medium

Components		Concentration (mmol)
$NaNO_3$		17.6
$K_2HPO4{\cdot}3H_2O$		0.23
$MgSO_4{\cdot}7H_2O$		0.3
$CaCl_2{\cdot}2H_2O$		0.24
Citric Acid		0.031
$MgNa_2EDTA{\cdot}H_2O$		0.0027
Ferric ammonium citrate		0.021
Na_2CO_3		0.19
Trace Metal Solution	H_3BO_3	0.046
	$MnCl_2{\cdot}4H_2O$	$9.15x10^{-3}$
	$ZnSO_4{\cdot}7H_2O$	$7.65x10^{-4}$
	$Na_2MoO_4{\cdot}2H_2O$	$1.61x10^{-3}$
	$CuSO_4{\cdot}5H_2O$	$3.2x10^{-4}$
	$CoCl_2{\cdot}6H_2O$	$2.1x10^{-4}$

ACKNOWLEDGEMENTS:

We are grateful to the Ibero-American network Cyted Renuwal, the ALGATEC-CM Project (P2018/BAA-4532) co-financed by the European Social Funds and the SETH Project (RTI2018-RTI2018-094399-A-I00).

DISCLOSURES:

The authors declare they have no conflicts of interest.

REFERENCES

Ahmad I. Z. (2022). The usage of Cyanobacteria in wastewater treatment: prospects and limitations. Letters in applied microbiology, 75(4), 718–730. https://doi.org/10.1111/lam.13587

El-Bestawy E. (2008). Treatment of mixed domestic-industrial wastewater using cyanobacteria. Journal of industrial microbiology & biotechnology, 35(11), 1503–1516. https://doi.org/10.1007/s10295-008-0452-4

Garlapati, D., Chandrasekaran, M., Devanesan, A., Mathimani, T., & Pugazhendhi, A. (2019). Role of cyanobacteria in agricultural and industrial sectors: an outlook on economically important byproducts. Applied microbiology and biotechnology, 103(12), 4709–4721. https://doi.org/10.1007/s00253-019-09811-1

Saeed, M. U., Hussain, N., Shahbaz, A., Hameed, T., Iqbal, H. M. N., & Bilal, M. (2022). Bioprospecting microalgae and cyanobacteria for biopharmaceutical applications. Journal of Basic Microbiology, 62(9), 1110–1124. https://doi.org/10.1002/jobm.202100445

Silva, C. P., Otero, M., & Esteves, V. (2012). Processes for the elimination of estrogenic steroid hormones from water: a review. Environmental pollution (Barking, Essex: 1987), 165, 38–58. https://doi.org/10.1016/j.envpol.2012.02.002

Zahra, Z., Choo, D. H., Lee, H., & Parveen, A. (2020). Cyanobacteria: Review of current potentials and applications. Environments, 7(2): 13.

CULTURE OF *Arthrospira platensis* IN MEDIA FORMULATED WITH COMMERCIAL GRADE NITROGEN SOURCES.

Xavier Álvarez-Montero[1], Ingrid Mercado-Reyes[2], Sandra Naranjo[1], Sandra Armijos[1], Nahir Dugarte[1], Mariela Valdiviezo[1]

[1] Universidad de las Fuerzas Armadas (ESPE), Sede Santo Domingo de los Tsáchilas, Santo Domingo 230101, Ecuador.
[2] LAB-BIOTEM S.A., Ceibos calle 17ava y transversa, Guayaquil, Ecuador.
Correspondence: xgalvarez@espe.edu.ec

SUMMARY

In this protocol, three culture media were formulated, and the nitrogen source variable was evaluated (M_1: NH_4NO_3, M_2: $(NH_4)_2SO_4$, and M_3: NH_2CONH_2) and its influence on growth parameters and the quality of the biomass of *Arthrospira platensis*, which were quantified spectrophotometrically. M_2 showed the best results compared to the standard Zarrouk culture medium, showing a higher rate of specific growth, protein fraction, and production of cyanobacterial biomass.

INTRODUCTION

The United Nations Department of Economic and Social Affairs (DESA) estimated a world population of 7.3 billion in 2015, increasing and is expected to increase to 9.7 billion in 2050 (UN DESA, 2015). Consequently, population growth brings an increase in the demand for food, which anticipates a challenge due to the limited availability of land suitable for cultivation (FAO, 2009). Although biotechnological tools currently exist to improve the yield of crops, the search for alternatives to obtain food in small areas of land, and in the shortest possible time has become a primary need (Pathak et al., 2018).

In this way, cyanobacteria are considered promising resources in the face of growing concern for food security and the global energy crisis and as a tangible solution for environmental sustainability (Zahra et al., 2020). Because these microorganisms have a shorter growth time relative to plants, they do not need to be grown on agricultural land, they can fix CO_2 from industries as the primary source of carbon, in addition to using different types of substrates for their growth (wastewater, industrial by-products, lignocellulosic material, commercial-grade fertilizers) (Perez-Garcia & Bashan, 2015).

Within the edge of cyanobacteria, there are various groups of microorganisms with the capacity to produce metabolites of biotechnological interest, due to their economic value and applications in multiple sectors such as pharmaceutical and nutraceutical, agricultural, bioremediation, wastewater treatment, bioplastics, and biofuels (Torrado, 2016).

Thus, the interest in cyanobacterial biomass production is increasing, although it is still necessary to establish production so that it satisfies global demand, covers production costs, and reduces biomass losses during production (Sido, 2019). There is currently a strategy that seeks to solve these problems, known as the Biorefinery of Photosynthetic Microorganisms, although still in the research and experimentation stage (Chisti, 2007). It should be noted that a large part of the studies carried out on photosynthetic microorganisms have focused on evaluating the effect of physical variations and its possible applications (Burgos-Rada et al., 2016; Silva-Benavides, 2016), however, the use of microbial biomass for energy purposes has had little industrial development.

Consequently, this protocol aims to evaluate the viability of formulating three culture media in seawater and the response to the nutritional requirements of *Arthrospira platensis.* Urea, ammonium nitrate, and ammonium sulfate will be used as a combined source of nitrogen and their impact on the quality of the biomass (growth parameters and biochemical composition) will be determined. The final nitrogen concentrations used were established as a function of the effective nitrogen content of the standard Zarrouk culture medium (Zarrouk, 1996), while the cofactors and elements required in the photosynthesis process according to the reviewed literature (Xiong et al., 2017).

MATERIAL AND METHODS

Microorganism

Arthrospira platensis (Figure 1) was obtained from the Biology Research Center of the University of Santiago (CIBUS) and kept in Zarrouk standard culture medium.

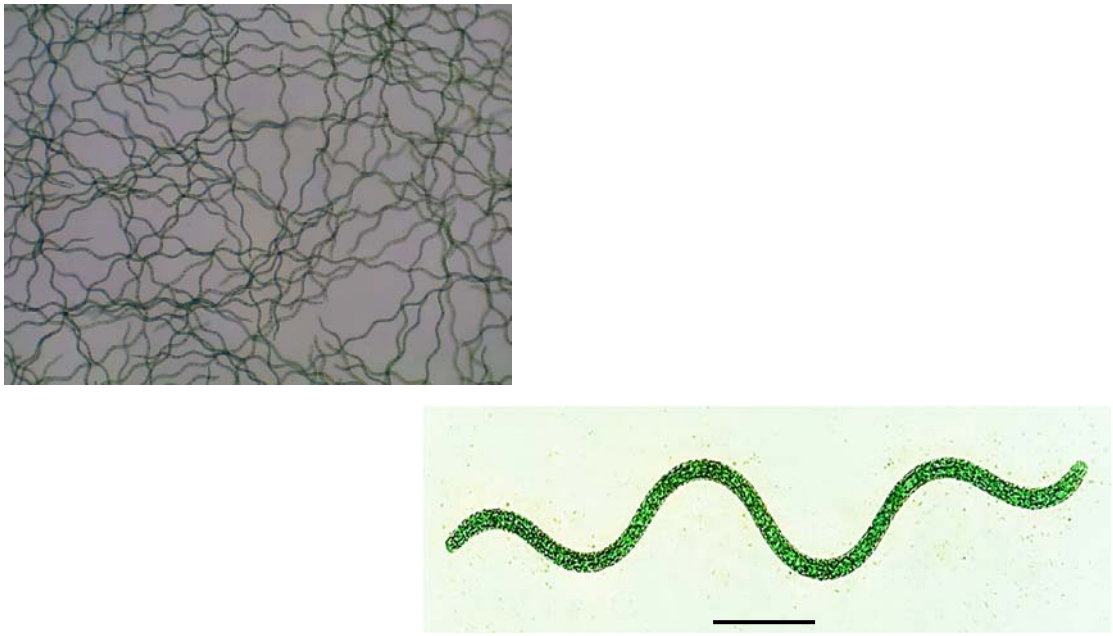


Figure 1. Light microscopy of *Arthrospira platensis* filament (10-40x). Scale: 100 μm.

Culture conditions and Formulation of Alternative Culture Media

Arthrospira platensis was grown in triplicate in *batch* culture in photobioreactors of 200 mL capacity, photoperiod 12 hours light / 12 hours dark, light irradiance of 164 µmol photon m^{-2} s^{-1}, a supply of continuous airflow, and addition of CO_2 pulses, to help regulate the pH (9.9 ± 0.3) of the culture media. Cultures were inoculated with an average chlorophyll-*a* concentration of 13 ± 0.20 mg L^{-1} and evaluated for seven days.

For the control, the Zarrouk culture medium was used, and the treatments consisted of culture media formulated with previously filtered and sterilized seawater, and macronutrients and micronutrients were added to support the growth of the cyanobacteria. Reconstituted seawater was used with K_2HPO4, $NaHCO_3$, the cofactors ($NiSO_4$, Na_2MoO_4, $FeSO_4$), and a combined nitrogen source (NH_4NO_3, $(NH_4)_2SO_4$, NH_2CONH_2), maintaining the effective nitrogen concentration of the Zarrouk medium, 29.4 mmol (see Table 1).

Table 1. Constitution of culture media with commercial grade nitrogen sources.

Treatment 1 (M_1)		Treatment 2 (M_2)		Treatment 3 (M_3)	
Composition	mmol	Composition	mmol	Composition	mmol
$NaHCO_3$	47.6	$NaHCO_3$	47.6	$NaHCO_3$	47.6
$(NH_4)NO_3$*	14.62	$(NH_4)_2SO_4$*	14.61	NH_2CONH_2*	14.65
K_2HPO_4	2.9	K_2HPO_4	2.9	K_2HPO_4	2.9
Na_2MoO_4**	0.00009	Na_2MoO_4**	0.00009	$NiSO_4$**	1.1 x 10^{-8}
$FeSO_4$**, ***	0,04	$FeSO_4$**, ***	0,04	$FeSO_4$**, ***	0.04

*Nitrogen source; **Cofactors of assimilation of compounds of combined nitrogen; ***Essential in the photosynthesis process.

The final nitrogen concentration of each commercial fertilizer used was like the primary nitrogen source of the Zarrouk culture medium ($NaNO_3$ 29.41 mmol: 29.41 mmol N). Thus, the nitrogen concentrations for NH_4NO_3, $(NH_4)_2SO_4$, and NH_2CONH_2, were 14.62 mmol, 14.61 mmol, and 14.65 mmol, respectively.

Determination of Growth Parameters

Growth was measured daily as chlorophyll-*a* content by the method of Talling & Driver (1963). The optical density of the methanolic extract was determined by UV-Vis spectrophotometry at 665 nm and the concentration of chlorophyll *a* was quantified by the following equation:

$$Chl - a\ (mg - L^{-1}) = DO_{665nm}\ x\ 13.9, \qquad (1)$$

where OD is the optical density (665 nm), and 13.9 a constant value.

Specific growth rate (μ) was determined from the results obtained from chlorophyll-*a* concentration, following the equation suggested in (Arredondo et al., 2017):

$$\mu = {\ln(N_2 - N_1)}/{t_2 - t_1}, \qquad (2)$$

where N_1 and N_2 represent the concentration of chlorophyll *a* in mg L^{-1} at times t_1 and t_2, expressed in days.

Dry weight was determined using the methodology (Zhu & Lee, 1997), dry filters were used to filter 10 mL of culture and 10 mL of 5 mM HCO_2NH_4 (Ammonium Formate) was added during filtering to remove salts from the culture medium. Filter samples were dried at 80°C for 24 h and weighed, while the amount of dry biomass (Pb) obtained per liter of culture was determined by the difference between filter with dry filtered sample and dry filter. Biomass productivity (g L^{-1} d^{-1}) was estimated by the amount of Pb obtained at the end of the study at times t1 and t2, in days, following the equation:

$$Pb: (X_2-X_1) / t_2-t_1 \qquad (3)$$

where X_1 and X_2 correspond to the biomass concentration (g L^{-1}) at times t1 and t2, respectively.

Determination of the Biochemical Composition of Biomass

Protein content was determined by the method (Lowry et al., 1951) modified by (Herbert et al., 1971). The subsequent reading was realized in a 750 nm spectrophotometer, using bovine serum albumin as standard for the calibration curve. Carbohydrates in the biomass are determined by the Sulfuric Phenol Method (Dubois et al., 1956), and they were read by spectrophotometry at 485 nm, using glucose as a standard. Lipids, according to the technique (Bligh & Dyer, 1959), to extract lipids from the biomass, and for the spectrophotometric measurement of the samples at 375 nm, using palmitic acid as standard, the simple Carbonization Method (Marsh & Weinstein, 1966) was followed.

Phycobiliproteins concentration was determined according to the methodology described by Bennet and Bogorad (1973). The concentration in ug mL^{-1} of Phycocyanin (PC) and Allophycocyanin (APC) was determined by UV-VIS spectrophotometry at 615 and 652 nm, applying the following equations:

$$PC = {OD_{615} - 0.474(OD_{652})}/{5.34}$$

$$APC = {OD_{652} - 0.208(OD_{615})}/{5.09}$$

Nitrogen assimilation analysis of the formulated media

At the end of the test, PO_4^{3-} and NH_4^+ concentration was measured based on the Indophenol Method (Bolleter et al., 1961), the NO_3^- content, by the Chromotropic Acid Method (West & Ramachandran, 1966), and NH_2CONH_2, by the Urease-Indophenol Method (McCarthy, 1970). Additionally, PO_4^{3-} was determined by the Vanado-Molybdate Method (Tandon et al., 1986). The concentrations were determined in the Lovibond MD600 / MaxiDirect photometer according to the respective protocols.

RESULTS

The growth of the cyanobacterium *A. platensis* for seven days is shown in Figure 2, where it is observed that its growth was significantly influenced by the type of nitrogen source used. The growth reflected as chlorophyll *a* concentration in the media formulated with different nitrogen sources showed promising results, where Medium 2 and Medium 3 did not have significant differences compared to the control (25.41 ± 0.78), with a maximum concentration of 24.87 ± 0.20 and 22.61 ± 0.39 mg L^{-1} Chl *a*, respectively. Regarding the specific growth rate, the medium formulated with $(NH_4)_2SO_4$ as a nitrogen source showed an increase of 14% compared to the Zarrouk culture medium, with μ values 0.963 ± 0.296 d^{-1} and 0.844 ± 0.154 d^{-1} for Medium 2 and control, respectively (Table 2).

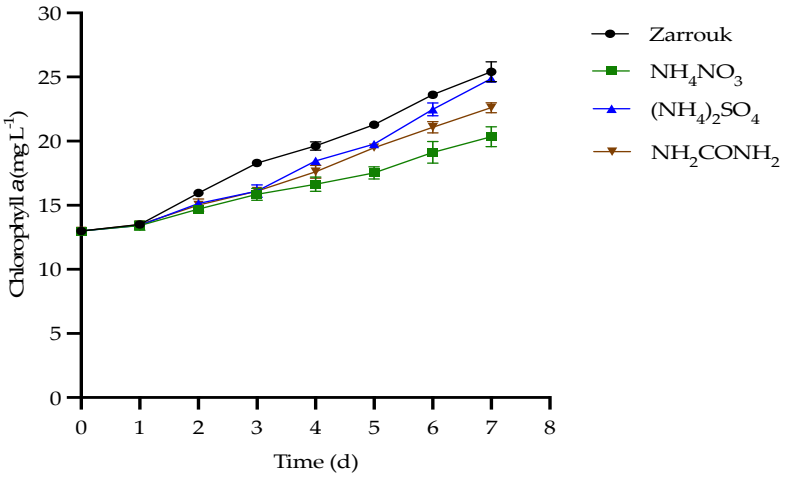


Figure 2. Chlorophyll-*a* content of *Arthrospira platensis* in batch culture under controlled laboratory conditions in photobioreactors, in standard Zarrouk culture medium and media formulated with seawater, different nitrogen sources, and other nutrients, at a time (d). Values are average ± SD (n = 3).

The growth measured as dry weight showed that the formulated media allowed the increase of the biomass produced in the three treatments, with values that exceed by 17, 38 and 29% (M_1, M_2, M_3) what is obtained with the Zarrouk medium (Figure 3, Table 2).

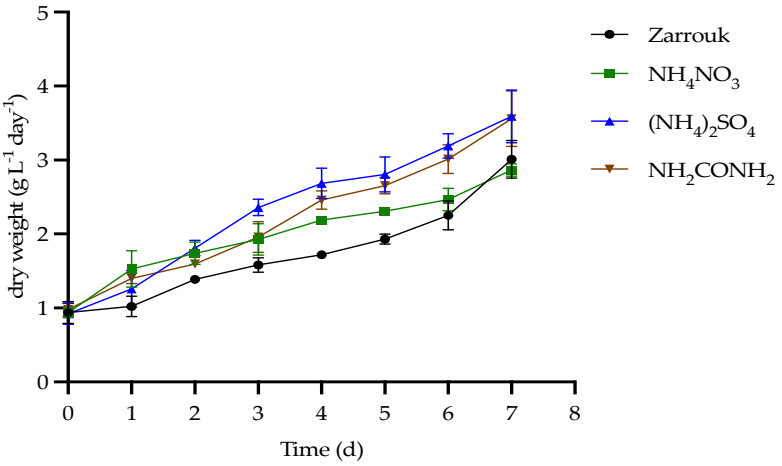


Figure 3. The dry biomass concentration of *Arthrospira platensis* was obtained in 7 days of culture, in Zarrouk standard medium and formulated media. Values are average ± SD (n = 3).

Table 2. Kinetic parameters of *Arthrospira platensis* grown in photobioreactors during seven days of culture in control medium and formulated media. Zarrouk: control culture medium; X_m: maximum chlorophyll-*a* concentration; μ: specific growth rate; DW: dry weight; Q_x: biomass productivity. Values are average ± SD (n = 3).

Culture medium	X_m (mg L^{-1})	μ (day^{-1})	DW (g L^{-1})	Q_x (g L^{-1} day^{-1})
Zarrouk	25.41 ± 0.78	0.844 ± 0.154	1.84 ± 0.62	0.43 ± 0.04
NH_4NO_3*	20.33 ± 0.78	0.460 ± 0.219	2.15 ± 0.45	0.41 ± 0.01
$(NH_4)_2SO_4$*	24.87 ± 0.20	0.963 ± 0.296	2.53 ± 0.78	0.51 ± 0.05
NH_2CONH_2*	22.61 ± 0.39	0.452 ± 0.115	2.38 ± 0.76	0.51 ± 0.05

*Plus cofactors of assimilation of compounds of combined nitrogen.

Determination of the Biochemical Composition of Biomass

All the media formulated with the different nitrogen sources presented a higher protein concentration than the control, obtaining percentages of 5, 27, and 15% more than the culture with the Zarrouk medium, with Treatment 1, 2, and 3, respectively (Figure 4b, Table 2). Formulated Medium 2 showed the highest protein concentration (420.10 ± 56.83 mg g^{-1}) with $(NH_4)_2SO_4$ as the nitrogen source.

Regarding the concentration of carbohydrates, all the treatments showed a lower content compared to the control, except for formulated Medium 3 with NH_2CONH_2 as a nitrogen source, with 22% more than the Zarrouk culture medium (Figure 4a, Table 3). The lipid fraction showed similar results, where all the treatments had a lower concentration compared to the control, and the formulated Medium 2 (NH_4NO_3) did not present significant differences concerning the Zarrouk medium.

Phycobiliproteins concentration in all treatments showed a lower proportion mg g^{-1} compared to control, for Phycocyanin was obtained: 90.58 ± 4.91, 67.82 ± 8.99, 75.57 ± 5.07 and 59.38 ± 3.43 mg g^{-1} for control, Medium 1, 2 and 3, respectively; while for Allophycocyanin was obtained 117.94 ± 6.87 mg g^{-1} for culture with Zarrouk medium, and 83.05 ± 10.93, 84.73 ± 8.25 and 51.20 ± 2.07 for Treatments 1, 2 and 3, as appropriate.

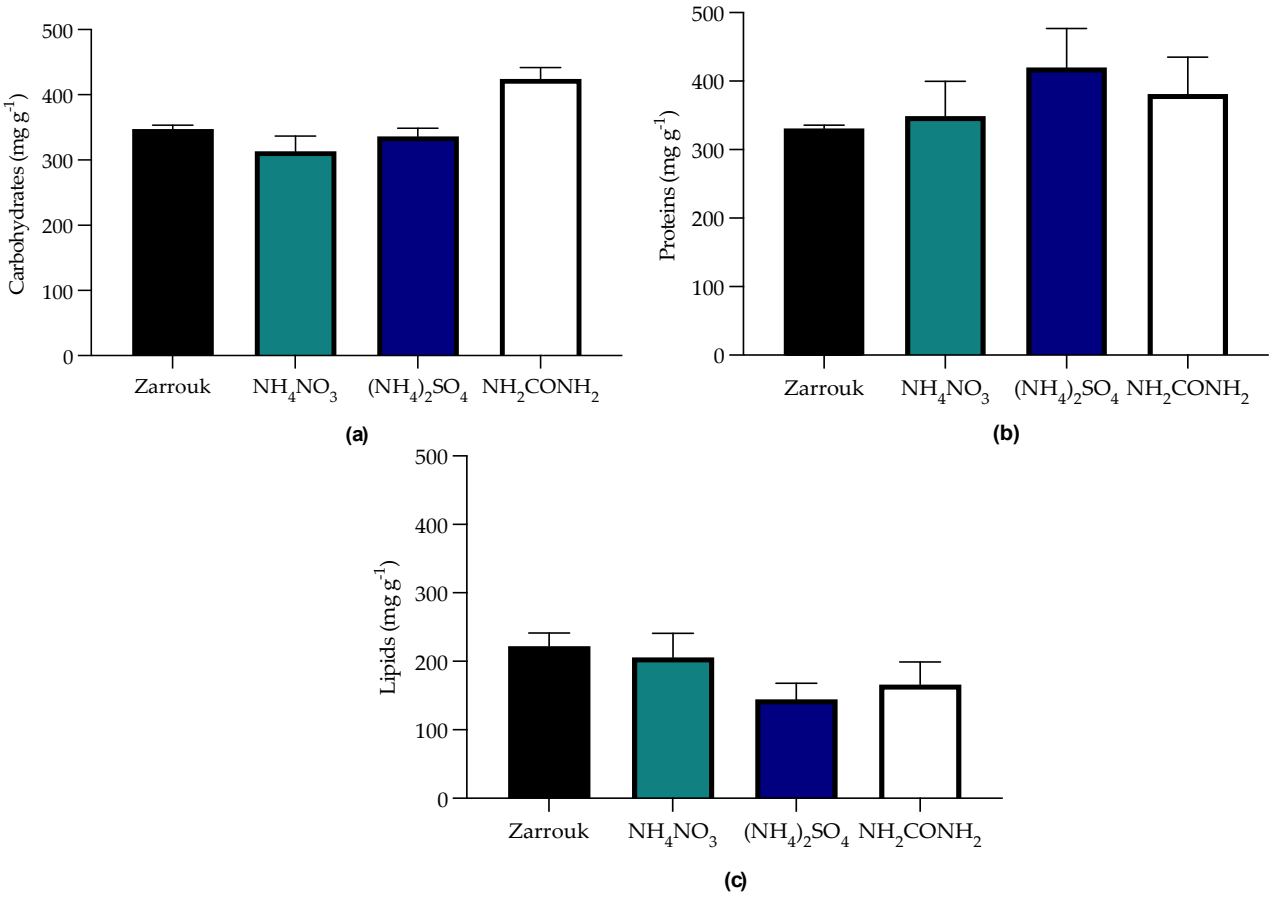


Figure 4. Biochemical composition of the biomass of *Arthrospira platensis*: (a) carbohydrates, (b) proteins, and (c) lipids content, obtained under controlled laboratory conditions, in Zarrouk standard medium and formulated media. Values are average ± SD (n = 3).

Table 3. Biochemical composition of *Arthrospira platensis* in Zarrouk medium (control) and medium consisting of seawater and agricultural fertilizers. Values are average ± SD (n = 3).

Culture medium	Carbohydrates (mg g^{-1})	Proteins (mg g^{-1})	Lipids (mg g^{-1})
Zarrouk	347.85 ± 5.52	331.48 ± 5.04	222.02 ± 18.91
NH_4NO_3*	313.56 ± 23.20	348.93 ± 50.65	205.56 ± 34.96
$(NH_4)_2SO_4$*	336.31 ± 12.54	420.10 ± 56.83	144.50 ± 23.16
NH_2CONH_2*	424.79 ± 16.90	381.50 ± 53.71	166.05 ± 32.90

*Plus cofactors of assimilation of compounds of combined nitrogen.

Nitrogen assimilation analysis of the formulated media

Considering the initial nitrogen concentration with which we worked, it was determined by its consumption that *A. platensis* was able to assimilate the supplemented nitrogen forms efficiently, percentages of assimilation between 95.26% and 96.33% were observed, higher percentages obtained in cultures with standard Zarrouk medium (Table 4). These results show the correct functioning of the enzymes participating in the nitrogen cycle. In addition, phosphate showed assimilation between 79 and 90%, higher than that obtained with the standard Zarrouk culture medium, and Medium 3 obtained the highest percentage of phosphorus removal (90.83 ± 6.42, as shown in Table 4).

Table 4. Initial and residual nitrogen concentration in the culture media with *Arthrospira platensis*. N_0 initial nitrogen concentration (mmol N); N_f final nitrogen concentration (mmol N). Values are average ± SD (n = 3).

Culture medium	N_0 (mmol N)	N_f (mmol N)	% Assimilation N	% Assimilation P
Zarrouk	29.41	1.43	95.15 ± 0.24	78.35 ± 4.56
NH_4NO_3	29.34	1.07	96.33 ± 0.06	79.22 ± 3.33
$(NH_4)_2SO_4$	29.30	1.19	95.93 ± 0.37	79.63 ± 3.45
NH_2CONH_2	29.29	1.30	95.26 ± 0.26	90.83 ± 6.42

DISCUSSION

Zarrouk medium was developed in 1966, since then, it has been used as a standard culture medium for the growth of *Arthrospira*. Therefore, three medium cultures with seawater were formulated, considering the essential nutritional requirements of *Arthrospira*. Seawater is supplemented with nutrients because its non-conservative elements, those that do not have a constant concentration but are influenced by variations in biotic components, the season of the year, and location, they were in minimum concentrations to support the growth of *A. platensis*.

The nitrogen source was supplied with commercial-grade agricultural fertilizers (NH_4NO_3; $(NH_4)_2SO_4$; NH_2CONH_2); as a phosphorous source, K_2HPO_4, a precursor element in amino acid synthesis, as a carbon source, $NaHCO_4$, essential for carbon metabolism, and $NiSO_4$, Na_2MoO_4, and $FeSO_4$, as essential cofactors to promote the enzymatic activity of the cyanobacterium.

The concentration of nitrogen sources for the formulation of the culture media varied depending on the type of nitrogen used (nitric, ammoniacal, amine): 12 mM NH_4NO_3 and $(NH_4)_2SO_4$, and 10 mM NH_2CONH_2. These low concentrations of

nitrogen are because under conditions of high pH (9.9 ± 0.2), typical of the *Arthrospira* habitat for being an alkalophilic cyanobacterium, nitrogen in the form of ammonia tends to transform into ammonia (NH_3).

On the other hand, 10 mM NH_2CONH_2 leads to the formation of 15 mM NH_3 in the culture medium (Mackerras & Smith, 1986). Based on previous tests, initial concentrations of 31.23 mM, 18.91 mM, and 41.62 mM, respectively, were reduced for NH_4NO_3, $(NH_4)\ 2SO_4$, and NH_2CONH_2, such as that for phosphorus to maintain the 10.1 ratios of Zarrouk culture medium (29.4 mM $NaNO_3$ and 2.9 mM K_2HPO_4).

Determination of Growth Parameters

The content of chlorophyll-*a* in the culture of *A. platensis* in seawater supplemented with nutrients, reported in this study shows higher values and, in less time, than that reported by Leema et al. (2010), where three media were formulated in seawater supplemented with $NaHCO_3$, K_2HPO_4, $NaNO_3$, $FeSO_4\ 7H_2O$, Fe_2EDTA, obtaining between 7.83 - 9.64 mg L^{-1} of chlorophyll-*a* in 25 days of culture. Similarly, the specific growth rate was higher (μ: 0.56-0.67 d^{-1}) than that estimated by Leema et al. (2010) (μ: 0.18-0.26 d^{-1}). Licett et al. (2014) also reported low contents of chlorophyll-*a* (0.043 g L^{-1}) using urea as a nitrogen source in addition to having a salinity of 15‰. This effect explains Lámela et al. (2000) is due to the increase in the pH gradient across the thylakoid membrane, causing the reduction of the photosynthetic rate and, thus, the decrease in the chlorophyll-*a* content.

The highest amount of dry biomass among the treatments with a light irradiance of 164 μmol photon m^{-2} s^{-1} was 2.21 g L^{-1} with $(NH_4)_2SO^4$, followed by 1.46 g L^{-1} with NH_4NO_3, and 1.38 g L^{-1} with NH_2CONH_2. On the other hand, in the study carried out by Murugan et al. (2014) in the cultivation of *Spirulina platensis* in seawater at different proportions (1:4; 2:4; 3:4), dry biomass of 1.52 g L^{-1}, 2.72 g L^{-1}, and 1.92 g L^{-1}, was reported, respectively. Furthermore, it is essential to note that the authors do not mention the light intensity used or the addition of CO_2 pulses, if applicable.

On the contrary, Vieira et al. (2001) in the culture of *Spirulina platensis* with different nitrogen sources, concentrations (10mM - 30mM - 50mM) and light intensity (25.65 μmol photon m^{-2} s^{-1}) reported 0.99 g L^{-1} of dry biomass with 10 mM NH_4NO_3, and 0.91 g L^{-1} of dry biomass with 10 mM NH_2CONH_2. It should be noted that the results in the present study in the culture with 12mM NH_4NO_3 and 10mM NH_2CONH_2 were higher results than those obtained by the authors mentioned above.

Determination of the biochemical composition of biomass

The highest protein concentration in cultures with NH_4NO_3 and $(NH_4)_2SO_4$ concerning NH_2CONH_2 , it is suggested that it could be due to the presence of the ammoniacal form in the elemental composition of fertilizers, since NH_4 is directly assimilated and directed to amino acid synthesis, therefore, energetically favorable for the cells. However, these protein percentages are low concerning the control. Low protein percentages were also reported by Madkour et al. (2012) in the culture of *Spirulina platensis* with NH_4NO_3 and NH_2CONH_2, concerning the control.

The higher content of carbohydrates and lipids with NH_2CONH_2, it is presumed may be due to the ability of urea to act as a source of nitrogen and carbon, likewise, ammonia (NH_3) product of the hydrolysis of urea is directly assimilated by the cell, favoring the acceleration of cellular metabolism and consequently, increasing lipid synthesis. At the same time, the highest lipid content with $(NH_4)_2SO_4$, the oxidized form of sulfur (SO^{4-2}), is used by photosynthetic microorganisms in the synthesis of sulfolipids (Schmidt, 1988). These differences in the biochemical composition of *A. platensis* are also mentioned in the works carried out by Ferreira et al. (2010) and Madkour et al. (2012).

Similarly, it is essential to note that some authors mention that this increase in the content of carbohydrates and lipids with NH_2CONH_2 is the product of an adaptive response to toxicity at high concentrations of it (Converti et al., 2006). On the other hand, it is also mentioned that the variable salinity causes physiological changes and therefore, different responses in the synthesis of macromolecules, such as lower protein content and higher carbohydrate and lipid content, leading to thickening of the cell wall to avoid dehydration as a protection mechanism against salt stress (Licett et al., 2014; Murugan et al., 2014), as is the case of the results obtained in this study.

CONCLUSIONS

Our results indicate that the filamentous cyanobacterium *Arthrospira platensis* can be produced using commercial-grade nitrogen fertilizers reconstituted in seawater, supplemented with a source of phosphorus, representing an exciting alternative in the sustainable production of biomass of this cyanobacterium, with an interesting biochemical composition, which could have potential uses in the fields of agriculture, aquaculture, and bioenergy. The different sources of combined nitrogen used in this investigation affected the metabolic activity of the microorganism, which was reflected in the growth parameters and the biochemical composition of the biomass.

ACKNOWLEDGEMENTS:

We are grateful to the Ibero-American network Cyted Renuwal.

DISCLOSURES:

The authors declare they have no conflicts of interest.

REFERENCES

Arredondo, B.; Voltolina, D.; Zenteno, T.; Arce, M.; Gómez, G. Métodos y herramientas analíticas en la evaluación de la biomasa microalgal, 2nd ed.; Centro de Investigaciones Biológicas del Noroeste: La Paz, México, 2017.

Bennett, A.; Bogorad, L. Complementary chromatic adaptation in a filamentous blue-green alga. J. Cell Biol. 1973, 58(2), 419–435. https://doi.org/10.1083/jcb.58.2.419

Bligh, E.; Dyer, W. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 1959, 37, 911–917. Doi: 10.1139/y59-099.

Bolleter, W.; Bushman, C.; Tidwell, P. Spectrophotometric determination of ammonia as indophenol. Anal. Chem. 1961, 33, 592–594. doi: 10.1016/j.biortech.2005.12.011.

Burgos-Rada, C.; Jiménez-Forero, J.; Ramírez-Merlano, J. Fertilizante comercial como medio de cultivo para clorofitas (*Desmodesmus opoliensis*) y su afectación en la cinética celular. Rev. Invest. Pecu. 2016, 147–148.

Chisti, Y. Biodiesel from microalgae. Biotechnol. Adv. 2007, 25, 294–306. doi: 10.1016/j.tibtech.2007.12.002.

Dubois, M.; Gilles, K.; Hamilton, J.; Rebers, P.; Smith, F. Colorimetric method for determination of sugars and related substances. Anal Chem. 1956, 28, 350–356. doi: 10.1021/ac60111a017.

FAO. La agricultura mundial en la perspectiva del año 2050. FAO – Foro de expertos de alto nivel: Como alimentar al mundo 2050, Roma, October 2009.

Herbert, D.; Phipps, P.; Strange, R. Chemical analysis of microbial cells. In Methods in Microbiology. Norris, J., Ribbons, D., Eds.; Academic Press; England, 1971.

Lowry, O.; Rosebrough, N.; Farr, A.; Randall, R. Protein measurement with the folin phenol reagent. J. Biol. Chem. 1951, 193, 265–275. doi: 10.1016/0304-3894(92)87011-4.

Marsh, J.; Weinstein, D. Simple charring method for determination of lipids. J. Lipid. Res. 1966, 7, 574–576.

McCarthy, J. A urease method for urea in seawater. Limnol. Oceanogr. 1970, 15, 309.

Pathak, J.; Rajneesh; Maurya, P.; Singh, S.; Häder, D.; Sinha, R. Cyanobacterial Farming for Environment Friendly Sustainable Agriculture Practices: Innovations and Perspectives. Front. Environ. Sci. 2018, 6, 7. doi: 10.3389/fenvs.2018.00007.

Perez-Garcia O., Bashan, Y. Microalgal heterotrophic and mixotrophic culturing for bio-refining: from metabolic routes to techno-economics. In Algal Biorefineries, Prokop, A., Bajpai, R., Zappi, M. Eds.; Springer: Cham, 2015; ISBN: 978-3-319-20200-6.

Sido, M. Exploring an Integrated Manure-Seawater System for Sustainable Cyanobacterial Biomass Production. Appl. Sci. 2019, 3888. doi: 10.3390/app9183888.

Silva-Benavides, A. Evaluación de fertilizantes agrícolas en la productividad de la microalga *Chlorella sorokiniana*. Agro. Mesoam. 2016, 27, 265-275. doi: 10.15517/am.v27i2.24361.

Talling, J.F., Driver, D. (1963) Some Problems in the Estimation of Chlorophyll-a in Phytoplankton. Proceedings of the Conference of Primary Productivity Measurement, Marine and Freshwater, University of Hawaii, Honolulu, Atomic Energy Commission TID-7633, 142-146.

Tandon, H.; Cescas, M.; Tyner, E. An acid-free vanadate-molybdate reagent for the determination of total phosphorus in soils. Soil Sci. Soc. Am. J. 1986, 32, 48-51. doi: 10.2136/sssaj1968.03615995003200010001.

Torrado, C. Cultivo de cianobacterias para la producción de bioplásticos en un fotobiorreactor tubular cerrado alimentado con digestato. Escola Tècnica Superior d'Enginyeria de Camins, Canals i Ports, 2016.

UN DESA. World Population Prospects: The 2015 Revision. Available online: https://www.un.org/en/development/desa/publications/world-population-prospects-2015-revision.html,2015.

West, P.; Ramachandran, T. Spectrophotometric determination of nitrate using chromotropic acid. Anal. Chim. Acta, 1966, 35, 317–324. doi: 10.1016/s0003-2670(01)81682-2.

Xiong, W.; Cano, M.; Wang, B.; Douchi, D.; Yu, J. The plasticity of cyanobacterial carbon metabolism. Curr. Opin. Chem. Bio. 2017, 41, 12–19. doi: 10.1016/j.cbpa.2017.09.004.

Zahra, Z.; Choo, D.; Lee, H.; Parween, A. Cyanobacteria: Review of Current Potentials and Applications. Environments 2020, 7, 13. doi: 10.3390/environments7020013.

Zarrouk, C. Contribution a Letude d'une Cyanophycee. Influence de Divers Factours Physiques. Et Chimiques sur la Croissance et la Phytosynthese do Spirulina maxima. Ph.D. Thesis, University of Paris, Paris, France, 1966.

Zhu, C.; Lee, Y. Determination of biomass dry weight of marine microalgae. J. Appl. Phycol. 1997, 9, 189–194. doi: 10.1023/A:1007914806640.

CULTURE OF MICROALGAE IN DAIRY WASTEWATER: PHYLOGENETIC ANALYSIS AND BIOCHEMICAL COMPOSITION

Ingrid Mercado-Reyes[1], Xavier Álvarez-Montero[2], Sandra Naranjo[2], Sandra Armijos[2], Nahir Dugarte[2], Mariela Valdiviezo[2]

[1] LAB-BIOTEM S.A., Ceibos calle 17 ava y transversa, Guayaquil, Ecuador.
[2] Universidad de las Fuerzas Armadas (ESPE), Sede Santo Domingo de los Tsáchilas, Santo Domingo 230101, Ecuador.
Correspondence: xgalvarez@espe.edu.ec

SUMMARY

New processes are required to transform the current production schemes and convert the conventional schemes into more sustainable circular processes. This protocol aims to solve these challenges based on wastewater from the dairy industry, an adequate culture medium has been formulated that contains 80% of wastewater and additional fertilizers. Next, a strain capable of growing in this culture medium at a high growth rate has been identified, with phylogenetic analysis confirming it as *Scenedesmus* sp. Using the proposed culture medium, a maximum productivity and protein concentration of 2.43 ± 0.63 g L^{-1} day^{-1} and 384.85 ± 34.06 mg g^{-1}, respectively, have been obtained. High removal of N, P, and COD was obtained, with 79.24 ± 6.26%, 77.14 ± 1.12%, and 90.66 ± 2.01%, respectively.

INTRODUCTION

The food industry, a key pillar of the food supply chain, is one of the largest water consumers and generators of residual effluents. It is estimated that the dairy industry alone consumes between 2 and 4 liters of water per kilogram of product, generating between 0.5 and 20.5 liters of wastewater. In addition, almost 2.5 times the volume of milk that is processed is generated as wastewater, either in the form of used washing or spillage, which makes it harmful to ecosystems if it does not receive adequate prior treatment (Álvarez et al. 2020; Bolognesi et al. 2020; Asgharnejad et al. 2021).

Due to the concentration of organic load and nutrients of this type of wastewater, biotreatments are a more practical option than other methods (Do et al. 2019). Phycoremediation is based on microalgal metabolism and its ability to adapt to different substrates as an advantageous system that reduces the cost of nutrients and freshwater consumption to produce biomass with high added value, favoring scale production processes and protecting ecosystems from the risk of eutrophication and excessive accumulation of CO_2 in the atmosphere (Chai et al. 2021; Geremia et al.

2021). The biomass generated with these systems has a variety of sustainable applications, such as the production of renewable fuels, biostimulants, biofertilizers, biohydrogen, bio-alcohols, bio-hydrocarbons, or commercial, based on bioactive products such as proteins, pigments, antioxidants, fatty acids, amino acids (Do et al., 2019; Wollmann et al. 2019).

Dairy wastewater has been studied as a potential substrate to produce microalgae biomass. Brar et al. (2019), cultivating *Chlorella pyrenoidosa*, *Anabaena ambigua*, and *Scenedesmus abundans* in dairy wastewater at 75% (v/v), observed that the strains used tend to increase the dry weight content minimizing external nutrient requirements. Produced biomass can be used to obtain bioenergy by estimating the theoretical potential of the species to produce biogas, as verified by Álvarez et al. (2020) when using biomass of *Arthrospira platensis* grown in dairy wastewater as a substrate in mesophilic anaerobic co-digestion with cattle manure.

The composition of the algal biomass obtained will depend on the features of each effluent, which will have a direct impact on the metabolism of the strain used. For example, Mercado et al. (2020) showed that dairy wastewater induces a more outstanding production of energy compounds, in addition to cellular morphological changes. They obtained that the lipid synthesis increases up to 51% of the total dry weight, representing 45% more lipid content regarding control.

Selection and identification of robust and productive strains is a major challenge when coupling with wastewater treatment processes. It must be considered that microalgae are highly diverse microorganisms because they are part of a polyphyletic group, making the traditionally used taxonomic classification and identification processes (phenotypic characterization) unviable to the broad criteria of subjectivity (Ancona-Canché et al. 2017). Molecular techniques based on the extraction and purification of cellular DNA are currently used, identifying specific regions of the strains using PCR to amplify the 16S rRNA region using universal primers for greater robustness.

Dairy wastewater is an essential source of nutrients that can be used as a sustainable alternative for microalgae cultivation with industrial applications of commercial interest in agriculture, energy, and animal nutrition. This research proposes the application of a native strain from Ecuador in the treatment of dairy wastewater to evaluate the fundamental role of microalgae in biotreatment systems, analyzing growth, productivity, biochemical composition, and nutrient removal efficiency. After evaluating growth parameters and biomass composition, molecular techniques based on the amplification of the ITS molecular marker are proposed to get more reliable results due to its high specificity in eukaryotic species. The biochemical composition of the biomass allows us to explore potential applications of produced

biomass and to estimate a possible business plan for further development. Thus, this work provides the basis of sustainable processes, both economic and environmental, to be developed as a complement to the dairy industry to make it more profitable.

MATERIAL AND METHODS

Microorganism

The microalga used in this research comes from the bank of microalgae and cyanobacteria strains of the Microbial Biotechnology Laboratory (Lab-Biotem S.A.) (Guayaquil, Guayas, Ecuador), isolated from the Jauneche Reserve - Pedro Franco Dávila Research Station (1°14'50.2" S; 79°39'34.5" W).

DNA barcode analysis

DNA extraction was performed using the modified protocol of Morin et al. (2010). Briefly, 5 mL of cell culture was centrifuged at 1200 rpm for 20 min, 500 µL of TES lysis buffer (100 mM Tris, pH 8.0: 10 mM EDTA and 2% SDS) was added to the precipitate, and followed by sonication at 20 kHz at 4°C for 20 min. The sonicated sample was transferred to a 1.5 mL Eppendorf tube, and 12.56 µL of Proteinase K was added following incubation at 37°C for 30 minutes. Then, 140 µL of 5 mol NaCl and 1/10 volume of 10% CTAB were added, and it was incubated again at 65 °C for 10 min. One volume of chloroform: isoamyl alcohol (24:1) was added, then the tube was placed on ice for 5 min and centrifuged again at 14,000 rpm for 10 min at 4°C. The supernatant was removed and transferred to a new 2 mL Eppendorf tube. Then, 225 µL of 5 mol ammonium acetate was added left on ice for 5 min, and centrifuged at 14,000 rpm for 5 min at 4°C. Subsequently, 0.5 volumes of isopropanol were added to the supernatant, and the mixture was left to react at -20 °C overnight. Finally, the tube was centrifuged at 14,000 rpm for 20 min at 4°C. The pellet obtained was washed with 1 mL of cold 70% ethanol and centrifuged again at 14,000 rpm for 5 min at 4°C. Then, the pellet was dried in a laminar flow chamber for a period of 10 to 15 min and resuspended in 200 µL of ultra-pure water.

According to the modified protocol of Ancona-Canché et al. (2017), DNA amplification was performed in a final volume of 10 µL for the PCR reaction prepared in triplicate. Each primer was used at a final concentration of 500 nmol: ITS1 (5′- TCC GTA GGT GAA CCT GCG G -3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). The following components were added per PCR reaction: 5 µL of GoTaq® Green Master Mix (1x), 2 µL of H_2O, and 2 µL of DNA. Finally, the tubes were placed in a thermal cycler, with a thermal of 95°C for 5 min, 35 cycles of 95°C for 30 s, 55°C for 1

min, 72°C for 2 min, and 72°C for 5 min. PCR products were sequenced commercially (Macrogen, Rockville, MD, USA) after purification of PCR products.

The analysis of all the sequences was performed using the Mega X software. Following alignment, the sequences were trimmed at both ends, obtaining a consensus sequence uploaded at GenBank. The sequence was blasted for similarity. Different accessions were used to perform phylogenetic analysis after alignment with MUSCLE from MEGA X. The maximum likelihood method was used with a bootstrap test of 1000 replicates according to the best model suggested by MEGA X (Kumar and Chaurasia, 2018).

Culture medium

The wastewater used as an alternative culture medium comes from the distribution channel of a dairy industry (*DIWW*) specializing in dairy products and gourmet cheeses with the following initial chemical characteristics: pH 5.5, 2.22 mmol of total nitrogen (NT), 0.20 mmol of phosphate, 3500 mg L^{-1} of O_2 concerning chemical oxygen demand (COD), as described in Table 1.

Table 1.- Characteristics of untreated dairy wastewater (*DIWW*) used as an alternative culture medium

Parameter	Value
pH	5.5
DQO	3500 mg O_2 L^{-1}
Macroelements	
Total nitrogen	2.2 mM
Phosphate (PO_4^{-3})	0.2 mM

The microalgae growth was evaluated in gradual concentrations of 10 to 80% of the residual water to reduce the adaptation time when starting the cultures. For the test control, the standard medium BG11 was used (Allen, 1968). For the treatments, residual water from the dairy industry (*DIWW*) was used at 80% and 20% seawater, with a nutritional correction with the Bayfolan® fertilizer (2.5 mL L^{-1}), whose composition is detailed in Table 2, reaching a final concentration of 18.26 mmol of total nitrogen and 1.59 mmol of P_2O_5. Both cultures, the control, and the treatments were performed in quadruplicate.

Table 2.- Composition of the liquid foliar fertilizer Bayfolan® 11-8-6 (N-P-K).

Elements/compounds	Concentration
Total nitrogen	6,43 mol
P_2O_5	0,63 mol
K_2O	0,74 mol
Fe	3,40 mmol
Mn	2,91 mmol
B	9,34 mmol
Mo	0,10 mmol

Culture conditions

The microalgae were grown discontinuously in bubble column photobioreactors of 300 mL with a culture volume of 200 mL, with an initial cell density of 1.5 x 10^7 cell mL^{-1}. The reactors were artificially illuminated with a photoperiod of 12 h light:12 h darkness, at an irradiance of 100 μmol photon m^{-2} s^{-1}, provided by white light LED lamps. Temperature of the culture was maintained at 24 ± 2°C. Mixing was provided by passing a continuous airflow of 0.1 v/v/min, it also allowed to keep the dissolved oxygen in the range of 100 ± 10% Sat.

The reactors were daily sampled to determine the biomass growth rate, whereas biochemical analyses were performed at the end of the batch culture. Various analytical methods were applied to determine the growth parameters and biochemical composition of the biomass through the daily determination of cell concentration and dry biomass and the quantification of carbohydrates, proteins, and lipids at the end of the trial. The percent uptake of total nitrogen and phosphorus was also evaluated to determine the effectiveness of removing nutrients from the medium.

Analytical methods

Cell concentration was determined daily, as described by Guillard and Sieracki (2005), using a Neubauer hemocytometer chamber using the following equation:

$$C = N \times 10^4 \times f \qquad \text{Equation (1)}$$

where C is the cell concentration (cell mL^{-1}), N is the average number of cells counted in 1 mm (0.1 μL), and F is the dilution factor.

The dry weight of the biomass (g L^{-1}) was determined with the methodology proposed by Zhu and Lee (1997). The total dry weight was determined by the difference between the final weight of the filter with the dry biomass and the initial weight of the dry filter.

The concentration of carbohydrates in the biomass was determined by the phenol-sulfuric method proposed by Dubois et al. (1956) in 3 steps. Finally, the samples were allowed to cool for 30 min, and the carbohydrate concentration was read using UV-VIS spectrophotometry at 485 nm, using glucose as standard.

The protein fraction of the biomass was determined by the method of Lowry et al. (1951), modified by Herbert et al. (1971). Finally, the protein concentrations of each sample are calculated by spectrophotometry at an absorbance of 750 nm. The data obtained are quantified by interpolating the bovine serum albumin (BSA) standard curve.

The concentration of biomass lipids was carried out by Bligh and Dyer method (1959). Finally, the method of Marsh and Weinstein (1966) is applied to determine lipid concentration later by spectrophotometry at 375 nm, using palmitic acid as standard.

At the end of the trial, 10 mL of sample was extracted from each culture for analysis. The total nitrogen concentration was determined with an adaptation of the persulfate digestion method (D´Elia et al. 1977), and the total phosphorus concentration was the vanadate-molybdate method (Tandon et al. 1968). The concentrations were measured in the Lovibond® MD600/MaxiDirect photometry equipment with standard protocols.

Biomass growth rate, productivity, and nutrients removal

The specific growth rate (μ) was calculated by applying the equation proposed by Arredondo et al. (2017), based on a daily estimate of cell density:

$$\mu = \frac{\ln (N_2 - N_1)}{t_2 - t_1} \qquad \text{Equation (2)}$$

where N_1 and N_2: cell concentration at times t_1 and t_2, respectively.

Based on the daily estimation of dry weight, biomass productivity (g L^{-1} d^{-1}) was determined using the equation proposed by Mohd Apandi et al. (2021):

$$Pb = \frac{X_2 - X_1}{t_2 - t_1} \qquad \text{Equation (3)}$$

where X_1 and X_2 correspond to the biomass concentration at times t_1 and t_2.

For the nutrient removal efficiency, the percentage difference between the initial and final concentrations of each culture was determined according to the parameters evaluated, following equation 4.

$$\mathrm{Re} = 100 - \frac{C_f \text{ x } 100}{C_i} \qquad \text{Equation (4)}$$

where C_f and C_i represent the final and initial concentrations of the nutrients evaluated.

RESULTS

Identification of selected strains

The strain to be produced was selected based on previous experience confirming the reliability of this strain to be produced in different wastewater types. PCR amplification was obtained using the primers ITS1/ITS4. After sequencing the PCR products, a consensus sequence was generated from three technical replicates using Mega X. The unique sequence contains 659 bp in length, harboring a partial sequence of the small and large subunit of ribosomal RNA, and a complete sequence for ITS1, the 5.8s rRNA gene, and ITS2. The sequence was deposited in the GenBank with accession code MW817087 (https://www.ncbi.nlm.nih.gov/nuccore/MW817087). Blast analysis was performed in the GenBank database, resulting in a range of 99% similarity with sequences of various isolates of the family Scenedesmaceae (Table 3). The phylogenetic tree performed using the MEGA X software by the nucleotide substitution method "Maximum likelihood" (K2 + G model) shows that several accessions from the genus *Scenedesmus* are in a clade with the *Scenedesmus* sp. isolate LBM-0020 (Figure 1).

Table 3.- Percentage of similarity of the ITS sequence of *Scenedesmus* sp. (GenBank accession code MW817087) obtained from Blast analysis.

Organism	Accession	Query cover	E-value	% Identity
Tetradesmus nygaardii	MT023665.1	100%	0.0	99.85
Acutodesmus nygaardii	JQ082320.1	100%	0.0	99.85
Scenedesmus sp.	MN604368.1	100%	0.0	99.70

The Scenedesmaceae family comprises a wide diversity of taxonomic groups, as in the genus *Scenedesmus*, where 124 species have been accepted (Guiry and Guiry,

2021). This diversity is mainly attributed to its high degree of phenotypic plasticity, making its morphological identification almost invalid, so the application of molecular tools through barcodes for gene analysis or a specific region allows species identification quickly and efficiently. However, no gene ensures 100% of the functionality of an ideal barcode, as it presents a high genetic variability between species (Zou et al. 2016).

In the present investigation, the molecular analysis of strain LBM-0020 was performed by PCR amplification and Sanger sequencing with the use of the ITS barcode (primers ITS1 and ITS4), resulting in the analysis of a partial sequence of the small subunit and large rRNA and a complete sequence for the ITS1 region, the 5.8s rRNA gene and ITS2. Bellemain et al. (2010) used various combinations of nuclear DNA ITS for fungal identification of environmental samples, showing that ITS1 and ITS4 regions amplify many sequences for plant species of different orders. However, these ITS regions show significant differentiation in amplifying the sequences causing mismatches and pairing erroneous sequences that cause an information bias. Sarwa and Verma (2017) studied the similarity of two closely related microalgae corresponding to *Scenedesmus* sp. and *Acutodesmus* sp. through molecular characterization with the amplification of their sequences with the primers ITS1 and ITS4, obtaining a similarity range of 99-97% between the two strains of microalgae, according to the NCBI database.

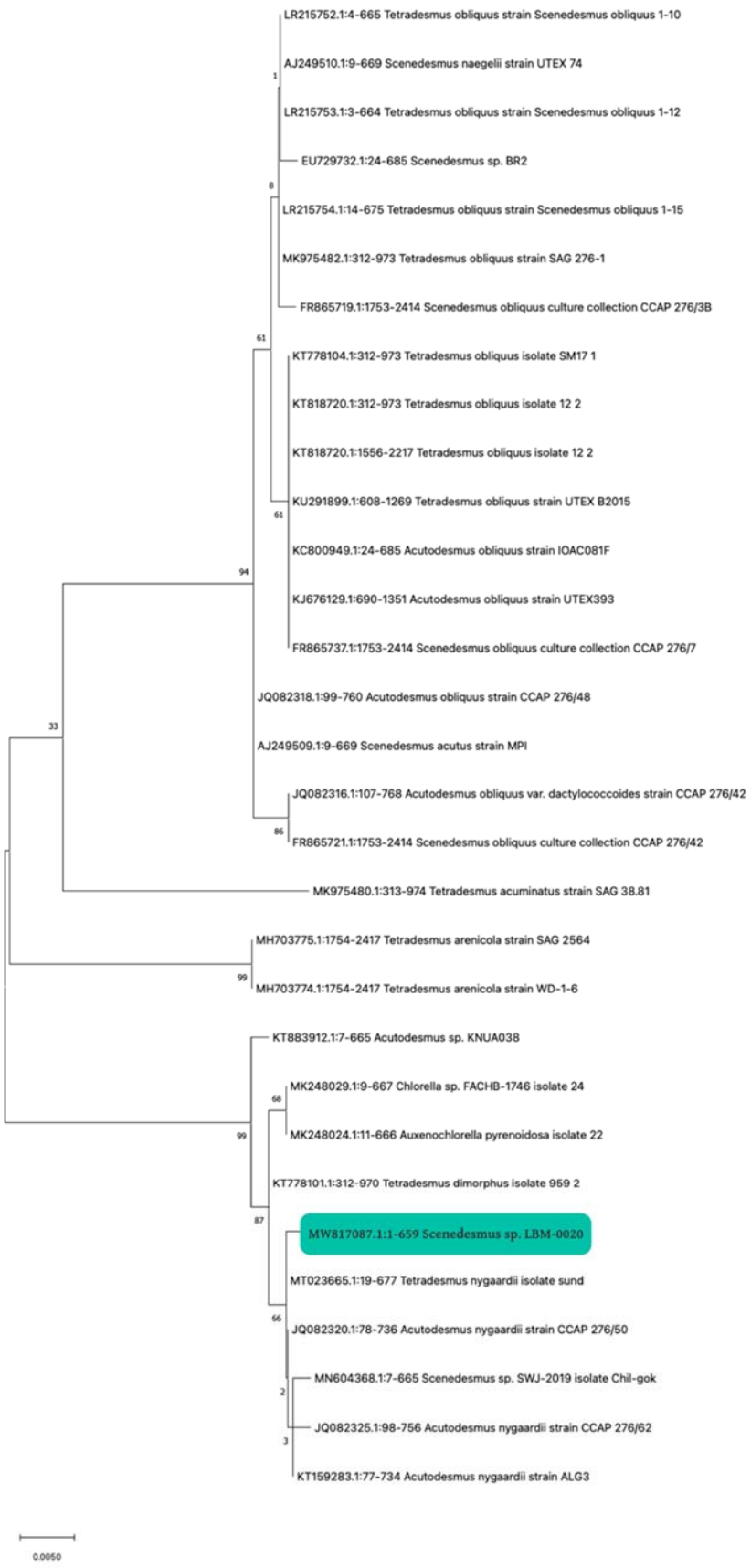


Figure 1.- Phylogenetic tree of the isolate *Scenedesmus* sp. with code LBM-0020. The *Scenedesmus* sp. isolate is indicated with a green square.

Biomass growth

The growth of *Scenedesmus* sp. on batch cultures is shown in

Figure **2**, results show that the culture in the residual water of the dairy industry at 80% showed a higher cell density (4.4 x 10^7 ± 1.4 x 10^6 cell mL^{-1}) compared to the control with the BG11 medium, where a maximum cell concentration of 4.3 x 10^7 ± 3.4 x 10^6 cell mL^{-1} was reached. However, it does not represent significant differences. During the exponential phase, the specific growth rate (μ) was determined based on the cell concentration, obtaining 1.467 ± 0.010 day^{-1} and 1.490 ± 0.012 day^{-1} in BG11 medium and *DIWW* 80%, respectively. Results from culture biomass concentration based on daily dry weight data reflect a 14% increase in the alternative medium in wastewater (2.43 ± 0.63 g L^{-1}) compared to that obtained in the standard medium BG11 (2.09 ± 0.77 g L^{-1}).

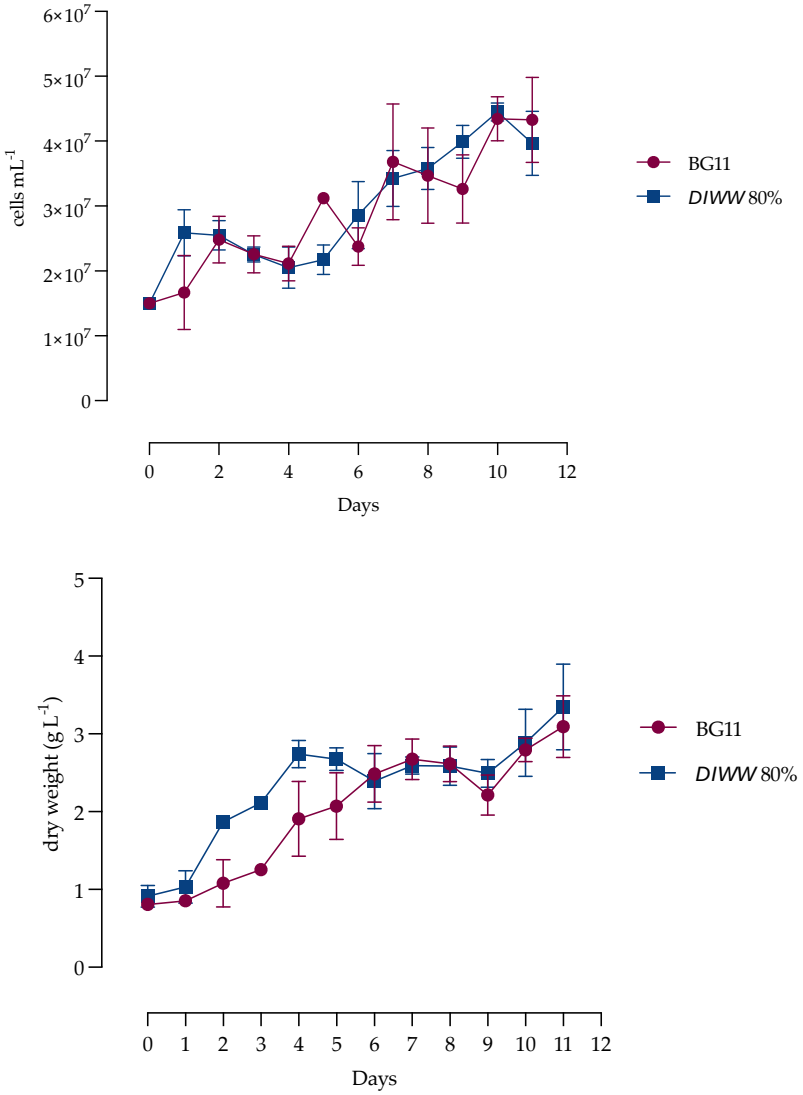


Figure 2.- Growth of *Scenedesmus* sp. measured as cell density and biomass dry weight in the standard medium BG11 (Control) and *DIWW* 80%. Data were analyzed with the mean ± SD of the four replicates.

The results confirmed the ability to use the native strain *Scenedesmus* sp. as a biotreatment mechanism for dairy wastewater due to its growth capacity in this type of substrates, reflected in the increase of up to three times the initial cell density after 11 days and without significant differences with the standard BG11 medium. It has been shown that agro-industrial wastewater is an alternative culture substrate that allows greater viability in producing microalgal biomass for industrial purposes. Our work showed an increase of 1.5% and 14% in the *DIWW* 80% in contrast to the BG11 medium regarding the specific growth rate and biomass productivity, decreasing the addition of micronutrients that increase the initial production costs. This is due to the characteristics of the wastewater used, which may vary according to the origin, place, and time of reception of the effluents, exclusively affecting the metabolic processes of each microalgal strain (Hussain and Bhattacharya, 2019). Singh et al. (2017) analyzed the cultivation of *Chlorella vulgaris* in urban wastewater (*UWW*) after the isolation of phytoplankton strains from the effluent by seeding on agar. They obtained an 11% increase in biomass productivity concerning the medium of BBM culture. However, the specific growth rate was higher in the control culture, 1.12 and 1.06 d^{-1}, for BBM and *UWW*, respectively, showing that the microalga used when cultivated in this type of effluent tends to increase its intracellular content reflected in the increase in biomass production although with a lower cell density. Mercado et al. (2020), when studying the growth of *Scenedesmus* sp. in 100% of dairy wastewater, observed results contrary to the previous investigation, having an increase of 5.5% in the specific growth rate in the treatment compared to the control.

It has been shown that agro-industrial wastewater is an alternative culture substrate that allows greater viability in producing microalgal biomass for industrial purposes. Our work showed an increase of 1.5% and 14% in the DIWW 80% in contrast to the BG11 medium regarding the specific growth rate and biomass productivity, decreasing the addition of micronutrients that increase the initial production costs. This is due to the characteristics of the wastewater used, which may vary according to the origin, place, and time of reception of the effluents, exclusively affecting the metabolic processes of each microalgal strain (Hussain and Bhattacharya, 2019). Singh et al. (2017) analyzed the cultivation of *Chlorella vulgaris* in urban wastewater (UWW) after isolation of phytoplankton strains from the effluent by seeding on agar. They obtained an 11% increase in biomass productivity concerning the medium of BBM culture. However, the specific growth rate was higher in the control culture, 1.12 and 1.06 d^{-1}, for BBM and UWW, respectively, showing that the microalga used when cultivated in this type of effluent tends to increase its intracellular content reflected in the increase in biomass production although with a lower cell density. Mercado et al. (2020), when studying the growth of *Scenedesmus* sp. in 100% of dairy wastewater,

observed results contrary to the previous investigation, having an increase of 5.5% in the specific growth rate in the treatment compared to the control.

Nutrients Removal

Microalgae use the nutrients of the medium as a culture substrate, which is why various low-cost media are being studied for scale production processes. Figure 3 shows that *Scenedesmus* sp. can take advantage of the nutrients in dairy wastewater, showing percentages of total nitrogen assimilation of 91.24 ± 3.09% and 79.24% ± 6.26% in the BG11 medium and *DIWW* 80%, as appropriate. Regarding total phosphate, there were percentages of assimilation greater than 75%, obtaining 77.44 ± 1.12% in *DIWW* 80% and 79.14 ± 3.18% in the BG11 medium.

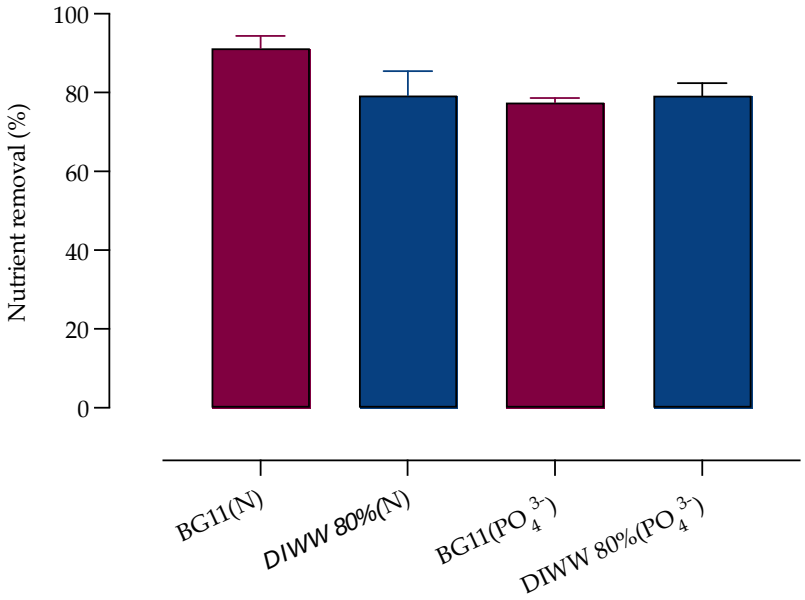


Figure 3.- Nutrient removal (NT, $PO4^{3-}$) in the culture of *Scenedesmus* sp. in BG11 medium and *DIWW* 80% were analyzed with the mean ± SD of the four replicates.

Regarding the removal of nutrients, in our research, there was better assimilation of nitrogen (91%) in the culture with BG11 due to the lower concentration of nitrates in the medium that allows the almost total absorption of these molecules, unlike the DIWW 80% medium, that having an additional source of nutrients (commercial fertilizer) makes it challenging to remove ammonium during the 11 days, obtaining the removal of 79.24% for total nitrogen and 77.44% for total phosphorus. It is inferred that if the culture time is expanded, *Scenedesmus* sp. can remove almost all the nutrients present in the culture medium with residual water while allowing greater biomass productivity. Choi (2016), when evaluating the cultivation of *Chlorella*

vulgaris in dairy wastewater for ten days, obtained removal percentages of 86% of total nitrogen and 66% of total phosphorus, like that obtained by Gatamaneni et al. (2020a), with assimilation percentages of NH4 of 86% and PO4-3 of 70% in the culture of *Chlorella variabilis* during 14 days with dilutions of 25% and 10% of the effluent. On the other hand, the specific composition of the wastewater to be used must be considered due to the presence of organic compounds (carbohydrates, animal fats, oils, surface-active agents, volatile compounds) and inorganic compounds (chlorides, heavy metals, sulfur) that can reduce the ability to metabolize nutrients. This is how Khalaji et al. (2021) observed that when cultivating *Chlorella vulgaris* with an initial density of 2.6 x 10^{-7} cells mL^{-1} in dairy wastewater at different concentrations (25%, 50%, 75%), the percentages of removal of nutrients such as nitrogen and phosphorus were less than 50%, due mainly to the presence of oils/fats and low concentrations of NO_3, NH_4, and PO_4^{-3}, factors that inhibit adequate nutrient removal.

Biochemical Composition

Microalgae are characterized by their ability to assimilate nutrients from wastewater and incorporate them into their metabolism to produce compounds with high-added value. In our investigation, promising results were obtained regarding the protein concentration of *Scenedesmus* sp., getting 5% more protein content in the *DIWW* 80% medium compared to the standard BG11 medium, with 366.72 ± 67.50 mg g^{-1} and 384.85 ± 34.06 mg g^{-1} of protein, for control and treatment, respectively (Figure 4).

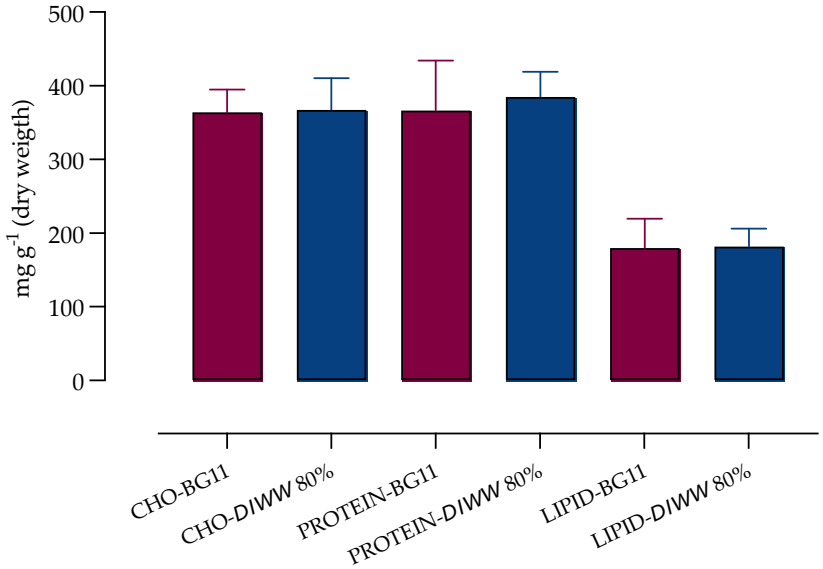


Figure 4.- Biochemical composition of the biomass of *Scenedesmus* sp. grown in BG11 culture medium and *DIWW* 80%. Data was measured in mg g^{-1} and analyzed with the mean ± SD of the four replicates.

In terms of carbohydrates and lipids, there were no significant differences between the culture in the BG11 medium and the culture in the residual water, showing the metabolic plasticity of *Scenedesmus* sp. to grow in dairy wastewater: the lipid fraction presented 180.10 ± 39.60 mg g^{-1} and 181.66 ± 24.65 mg g^{-1}, while the carbohydrate concentration remained at 364.26 ± 30, 79 mg g^{-1} and 367.69 ± 42.53 mg g^{-1}, for control and treatment, respectively (Tukey's test $P \leq 0.05$).

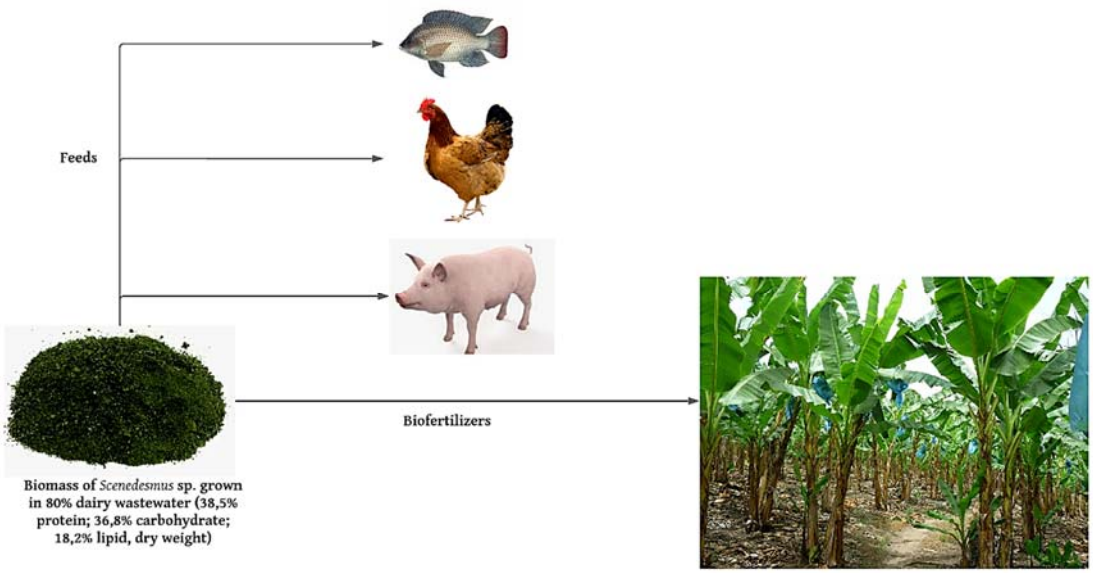
Using other sources of nutrient supply can also alter the flow of assimilated energy towards the main biomolecules present in the microalgal biomass, as in the present study, where 37%, 38%, and 18% production of carbohydrates, proteins, and lipids, as appropriate. De Sousa Oliveira et al. (2021) evaluated the batch culture of *Chlorella* sp. in pig wastewater in high-rate algal ponds (HRAP). Having at the end of the trial, 26%, 46%, and 25% of the fraction of carbohydrates, proteins, and lipids, respectively, were similar to what was obtained in our research, mainly due to the presence of ammonium salts and other nitrogenous compounds in these effluents that allow the design of various types of amino acids that bind to peptide sites, making proteins of different sizes. In other works, using a variety of microalgae from the Scenedesmaceae family, a higher protein fraction has also been obtained, directing the use of biomass for animal feed purposes or as biostimulants. Do et al. (2019) used *Tetradesmus obliquus* and *Desmodesmus* sp. in the culture in previously filtered municipal wastewater for 11 days, with protein percentages of 34% and 40% for both species, respectively, unlike those of both species, the lipid and carbohydrate fraction with values between 17 and 27%.

The type of light used in the experiment can also induce higher protein production. Thus, cool white fluorescent LED lights favor higher protein concentration. For example, Gatamani et al. (2020b) studied the effects of light quality on physiological and biochemical properties in the cultivation of *Chlorella variabilis* and *Scenedesmus obliquus* in dairy wastewater. They showed protein percentages more significant than 50% by weight dryness of biomass by using cold white, fluorescent light, unlike energetic compounds that reached concentrations of less than 35%. This is because white lights generally promote a more significant accumulation of compounds derived from nitrogen metabolism and higher photosynthetic efficiency, growth rate, and electron transport rate. However, this can vary according to the substrate culture and strain (Singh and Singh, 2015).

Potential applications

The biomass obtained from *Scenedesmus* sp. has an interesting biochemical composition that allows a variety of sustainable applications of commercial interest in

agriculture, animal nutrition, and energy, such as the production of biofertilizers or in animal feed, due to the presence of bioactive compounds as shown in the diagram below.



CONCLUSIONS

The results obtained during the current investigation show that the native microalgae *Scenedesmus* sp. grows favorably in dairy wastewater with promising results in terms of growth parameters and biochemical composition of the biomass, reaching higher percentages of the protein fraction that allows directing the use of biomass with fines in animal feed. Furthermore, *Scenedesmus* sp. has the metabolic capacity to assimilate, at high rates, the organic compounds present in the wastewater, an exceptional alternative in the biotreatment processes of industrial wastewater.

ACKNOWLEDGEMENTS:

We are grateful to the Ibero-American network Cyted Renuwal.

DISCLOSURES:

The authors declare they have no conflicts of interest.

REFERENCES

Allen, M. M., 1968. Simple Conditions for Growth of Unicellular Blue-Green Algae on Plates. *J. Phycol.*, *4*(1), 1–4. https://doi.org/10.1111/j.1529-8817.1968.tb04667.x

Álvarez, X., Arévalo, O., Salvador, M., Mercado, I., & Velázquez-Martí, B., 2020. Cyanobacterial Biomass Produced in the Wastewater of the Dairy Industry and Its Evaluation in Anaerobic Co-Digestion with Cattle Manure for Enhanced Methane Production. *Processes*, 8(10), 1290. doi:10.3390/pr8101290

Ancona-Canché, K., López-Adrián, S., Espinosa-Aguilar, M., Garduño-Solórzano, G., Toledano-Thompson, T., Narváez, J., y Valdez-Ojeda, R., 2017. Molecular phylogeny and morphologic data of strains of the genus *Coelastrella* (Chlorophyta, Scenedesmaceae) from a tropical region in North America (Yucatan Peninsula). *Bot. Sci.*, 95 (3): 527-537. https://doi.org/10.17129/botsci.1201

Arredondo, B.; Voltolina, D.; Zenteno, T.; Arce, M.; y Gómez, G., 2017. *Métodos y Herramientas Analíticas en la Evaluación de la Biomasa Microalgal*, 2nd ed. Centro de Investigaciones Biológicas del Noroeste. La Paz: México.

Asgharnejad, H., Khorshidi Nazloo, E., Madani Larijani, M., Hajinajaf, N., & Rashidi, H., 2021. A comprehensive review of water management and wastewater treatment in food processing industries in the framework of the water-food-environment nexus. *Compr. Rev. Food Sci.*, 20(5), 4779–4815. doi:10.1111/1541-4337.12782

Bligh, E.G.; & Dyer, W.J., 1959. A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physio*l., 7: 911–917.

Bolognesi, S., Cecconet, D., & Capodaglio, A. G., 2020. Agro-industrial wastewater treatment in microbial fuel cells. Integrated Microbial Fuel Cells for Wastewater Treatment, 93–133. doi:10.1016/b978-0-12-817493-7.00005-9

Brar, A., Kumar, M., & Pareek, N., 2019. Comparative Appraisal of Biomass Production, Remediation, and Bioenergy Generation Potential of Microalgae in Dairy Wastewater. *Front. Microbiol.*, 10. doi:10.3389/fmicb.2019.00678

Chai, W. S., Tan, W. G., Halimatul Munawaroh, H. S., Gupta, V. K., Ho, S.-H., & Show, P. L., 2021. Multifaceted roles of microalgae in the application of wastewater biotreatment: A review. *Environ. Pollut.*, 269, 116236. doi:10.1016/j.envpol.2020.116236

Choi, H-J., 2016. Dairy wastewater treatment using microalgae for potential biodiesel application. *Environ. Eng. Res.*, 21(4): 393-400. https://doi.org/10.4491/eer.2015.151

Choi, Y.-K., Jang, H. M., & Kan, E., 2018. Microalgal Biomass and Lipid Production on Dairy Effluent Using a Novel Microalga, *Chlorella* sp. Isolated from Dairy Wastewater. *Biotechnol. Bioprocess Eng.*, 23(3), 333–340. doi:10.1007/s12257-018-0094-y

D'Elia, C.F.; Steudler, P.A.; & Corwin, N., 1977. Determination of total nitrogen in aqueous samples using persulfate digestion1. *Limnol. Oceanogr*. 22:760–764.

Do, J., Seung-Woo, J., Il-Sup, K., Ho, N., Jae, L., Han, K., y Ho-Sung, Y., 2019. A Feasibility Study of Wastewater Treatment Using Domestic Microalgae and Analysis of Biomass for Potential Applications. *Water*, 11(2294). https://doi.org/10.3390/w11112294.

Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; & Smith, F., 1956. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem*, 28:350–356.

Geremia, E., Ripa, M., Catone, C. M., & Ulgiati, S., 2021. A Review about Microalgae Wastewater Treatment for Bioremediation and Biomass Production—A New Challenge for Europe. *Environments*, *8*(12), 136. https://doi.org/ 10.3390/environments8120136

Guillard, R. R., & Sieracki, M. S., 2005. Counting cells in cultures with the light microscope. Algal Culturing Techniques, 239-252.

Guiry, M.D.; & Guiry, G.M., 2021. AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org

Herbert, D.; Phipps, P.; & Strange, R., 1971. Chapter III Chemical Analysis of Microbial Cells. *Methods Microbiol*., 5: 209–344.

Hussain, A., & Bhattacharya, A. (Eds.)., 2019. Advanced Design of Wastewater Treatment Plants: Emerging Research and Opportunities: Emerging Research and Opportunities. IGI Global.

Kumar, M., & Chaurasia, N., 2018. Molecular characterization of freshwater microalgae and nutritional exploration to enhance their lipid yield. *3 Biotech*, 8:238. https://doi.org/10.1007/s13205-018-1248-5

Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; & Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem*., 193: 265–275.

Marsh, J.B.; & Weinstein, D.B., 1966. Simple charring method for determination of lipids. *J. Lipid Res*.7: 574–576.

Mercado, I.; Álvarez, X.; Verduga, M.; & Cruz, A., 2020. Enhancement of Biomass and Lipid Productivities of *Scenedesmus* sp. Cultivated in the Wastewater of the Dairy Industry. *Processes*, 8, 1450. https://doi.org/10.3390/pr8111458

Mohd Apandi, N., Muhamad, M. S., Radin Mohamed, R. M. S., Mohamed Sunar, N., Al-Gheethi, A., Gani, P., & A Rahman, F., 2021. Optimizing of microalgae *Scenedesmus* sp. biomass production in wet market wastewater using response surface methodology. *Sustainability*, 13(4), 2216.

Morin, N., Vallaeys, T., Hendrickx, L., Natalie, L., & Wilmotte, A., 2010. An efficient DNA isolation protocol for filamentous cyanobacteria of the genus *Arthrospira*. *J. Microbiol. Methods*., 80, 148–154. https://doi.org/10.1016/j.mimet.2009.11.012

Singh, R., Birru, R., and Sibi, G., 2017. Nutrient removal efficiencies of *Chlorella vulgaris* from urban wastewater for reduced eutrophication. *J. Environ. Prot. Sci.,* 8(1): 11. https://doi.org/10.4236/jep.2017.81001

Tandon, H.L.S.; Cescas, M.P.; & Tyner, E.H., 1968. An Acid-Free Vanadate-Molybdate Reagent for the Determination of Total Phosphorus in Soils. *Soil Sci. Soc. Am. J*., 32: 48–51.

Wollmann, F., Dietze, S., Ackermann, J., Bley, T., Walther, T., Steingroewer, J., & Krujatz, F., 2019. *Microalgae wastewater treatment: Biological and technological approaches. Eng. Life Sci.,* doi:10.1002/elsc.201900071

Zhu, C.J.; Lee, Y.K., 1997. Determination of biomass dry weight of marine microalgae. *Environ. Biol. Fishes*, 9: 189–194.

Zou, S., Fei, C., Wang, C., Gao, Z., Bao, Y., He, M., and Wang, C., 2016. How DNA barcoding can be more effective in microalgae identification: a case of cryptic diversity revelation in *Scenedesmus* (Chlorophyceae). *Sci Rep*, 6(36822). https://doi.org/10.1038/srep36822

EXTRACTION AND QUANTIFICATION OF MYCOSPORINES FROM *Chlorella vulgaris* FOR SUNSCREEN FORMULATION

Juan Sandoval[1], Kelly Naranjo[1], Laura Casas[1]
[1] Universidad de América, EcoCampus de Los Cerros, Avenida Circunvalar No. 20-53, Bogotá D.C., Colombia
Correspondence: juan.sandoval@profesores.uamerica.edu.co

SUMMARY

This technical document outlines the process of extracting and quantifying mycosporines from the microalgae *Chlorella vulgaris* as part of a project to formulate sunscreen. The method details the extraction of mycosporines, which can be used in the production of sunscreens protecting against harmful UV rays. The protocol covers the extraction and quantification of total mycosporines without discrimination of types. Additionally, the document discusses the potential factors that can influence the results and guides expressing the findings.

INTRODUCTION

The *Semillero Procycles* at the University of America has been conducting research to evaluate the use of the microalgae *Chlorella vulgaris* for various applications, including the removal of pharmaceuticals from surface waters (Ávila-Cújar et al., 2021). The recent focus has been on producing a sunscreen based on microalgae. A critical step in this process involves extracting the metabolites responsible for conferring sun protection properties to the final product, known as UV filters, which absorb harmful UV radiation while remaining transparent in the visible light range.

Mycosporines (MAAs) are nitrogenous compounds found in some microalgae when exposed to high light intensities. They possess low molecular weight, water-solubility, and stability, making them excellent candidates for therapeutic, antioxidant, and UV-absorbent properties (Raj et al., 2021).

REAGENTS

- Distilled water
- 90% acetone
- Silica oxide

PROTOCOL

1. **Biomass Preparation:**

- Centrifuge the microalgal biomass at 1500 rpm for 15 minutes.
- Wash the biomass with distilled water to remove excess salts and measure its salinity using a conductivity meter.
- Dry the biomass in a ventilated desiccator at 65°C for 12 hours.
- Macerate the dried biomass and store it at -30°C for 72 hours to preserve its properties.
- Obtain wet biomass samples of the microalgae and agitate them with glass beads at 100 rpm for 55 minutes to assess cellular lysis.
- Separate the liquid fraction from the glass beads by filtration and centrifuge it at 4000 rpm for 30 minutes to obtain the extract, which should be lyophilized for better storage conditions (Bertoloni, 2022).

2. **Mycosporines Extraction:**

- Weigh 10 mg of the sample and 10 mg of silica oxide, grind them in a mortar for 5 minutes, and add them to test tubes with 4 mL of the polar solvent for extraction, such as 90% acetone (Outon, 2019). Alternatively, 95% ethanol can also be used.
- Store the samples at -20°C for 24 hours in complete darkness, then centrifuge them for 20 minutes, remove the precipitate, and transfer the supernatant to another test tube (Outon, 2019).

RESULTS

To quantify mycosporines, use a spectrophotometer to determine the optical density of the supernatant at a wavelength between 300-750 nm for each duplicate sample. Apply the following equation (Huang et al., 2016):

$$Total\ MAAs\ content = \frac{12{,}25\ A_{663{,}6} - 2{,}55\ A_{646{,}6}\ (mg * L^{-1})}{peso\ seco\ (g * L^{-1})} * \frac{A_{MAAS}}{A_{chla}} * 1000$$

Where:

- $A_{663,6}$ y $A_{646,6}$ = Absorbance of the extract at these wavelengths.
- A_{MAAS} y A_{chla} = Area under the curve at wavelengths 319-350 nm and 630-680 nm, respectively. Use a program like Originpro® to determine the area under the curve.

EXPRESSION OF RESULTS:

The results are expressed as micrograms of MAAs per gram of microalgal biomass (μg MAAs.g^{-1} biomass).

DISCUSSION

As noted by Outon (2019), in addition to selecting the microalgal strain and stress factors, variations in temperatures, cooling and freezing times, and the choice of solvent (including 100% methanol) can influence the obtained results. Accurate calibration of the spectrophotometer is crucial to avoid errors in absorbance determination and area under the curve calculations.

ACKNOWLEDGMENTS

To the Semillero Procycles, from Universidad de América for its constant support.

REFERENCES

Ávila-Cújar, L. C., Sotelo-Torres, B. E. y Sandoval-Herrera, J. A. (2021). Selección y comparación de las principales tecnologías para la remoción de compuestos farmacológicamente activos: una revisión. *Gestión y Ambiente*, 24(Supl3), 59–72. https://doi.org/10.15446/ga.v24nSupl3.97265

Bertoloni, B. (2022). *In vitro photoprotective attributes from the Chlorella vulgaris cultivated in photobioreactor*. [Trabajo de fin de Maestría en Fármacos y Farmacêuticos. Universidad de São Paulo] 2022.

Díaz-Cruz, M. S., & Barceló, D. (2009). Chemical analysis and ecotoxicological effects of organic UV-absorbing compounds in aquatic ecosystems. *TrAC Trends in Analytical Chemistry*, 28(6), 708-717.

Huang, J.J., Bunjamin, G., Teo, E.S. (2016). An enclosed rotating floating photobioreactor (RFP) powered by flowing water for mass cultivation of photosynthetic microalgae. *Biotechnol Biofuels* 9, 218. https://doi.org/10.1186/s13068-016-0633-8

Outón Porras, J. (2019). *Diseño y validación de un método no invasivo para la determinación del factor de protección de cremas solares. Fabricación de una crema a partir de microalgas y el diseño de su proceso de producción*. [Trabajo de grado para Ingeniería Química y Biotecnología. Universidad de Cádiz]

Raj, S., Kuniyil, A. M., Sreenikethanam, A., Gugulothu, P., Jeyakumar, R. B., & Bajhaiya, A. K. (2021). Microalgae as a source of mycosporine-like amino acids (MAAs); advances and future prospects. *International Journal of Environmental Research and Public Health*, 18(23), 12402.

EXTRACTION AND QUANTIFICATION OF BETA-CAROTENES FROM *Chlorella vulgaris* USING SPECTROPHOTOMETRIC METHOD

[1]Juan Sandoval[1], Kelly Naranjo[1], Laura Casas[1]
[1] Universidad de América, EcoCampus de Los Cerros, Avenida Circunvalar No. 20-53, Bogotá D.C., Colombia
Correspondence: juan.sandoval@profesores.uamerica.edu.co

SUMMARY

This technical document describes the process of extracting and quantifying beta-carotenes or carotenoids β from the microalgae *Chlorella vulgaris* using the spectrophotometric method. The method involves biomass separation, extraction, and quantification of carotenoids, with the results expressed in total content without discrimination of types. Additionally, alternative methods are discussed along with the key factors affecting determination accuracy.

INTRODUCTION

Beta-carotenes, or carotenoids β, comprise a family of 700 pigments responsible for the golden color of the skin (Biehler et al., 2009). They also play a vital role in capturing radiation, filtering ultraviolet rays, inhibiting free radical formation, capturing oxygen atoms, and preventing lipid oxidation in microalgae (Del Campo et al., 1999). Studies have shown their health benefits, including skin protection against sunburn and radiation, preventing photoaging and other sun-related skin problems (Gebregziabher et al., 2021). Beta-carotenes are essential pigments found in the microalgae *Chlorella vulgaris*. These pigments are responsible for the golden color of the skin and offer various health benefits, making their extraction and quantification crucial for research purposes.

REAGENTS

- Distillated water.
- Acetone at 90%.

PROTOCOL

1. Biomass Separation:

The solid biomass is separated from the culture medium by centrifugation at 3000 rpm for 15 minutes (Corzo Piñeros et al., 2019). It is recommended to store the samples overnight at temperatures between -14 °C and -18 °C. Before testing, the samples should be vigorously shaken, but this step is only necessary if the test is not conducted immediately after biomass separation.

2. Carotenoid Extraction:

- Weigh 0.2 g of previously treated microalgal biomass using a balance.
- Transfer the biomass to a 15 mL Falcon tube containing 5-7 mL of the extraction solution (0.1% BHT in acetone).
- Agitate the mixture using a vortex for 30 seconds.
- Subject the sample to ultrasonication for 10 minutes.
- Centrifuge the sample at 10,000 rpm for 10 minutes.
- Transfer the obtained supernatant to a 25 mL volumetric flask and repeat the process until the matrix is decolored.
- Finally, adjust the volumetric flask to the mark with the extraction solution (Ibanez, 2018).

RESULTS

- Weigh 10 mg of the sample and dissolve it in acetone to make up the volume in a 25 mL volumetric flask.
- Measure the absorbance in the range of 400 to 500 nm using a spectrophotometer with acetone as the blank to identify the maximum absorption wavelength.
- Calculate the concentration of beta-carotene in μg/mL using the following equation (Ibanez, 2018):

$$C_{\text{carotenoids }\beta} = \frac{(A_{MAX} * M * 1000)}{(\varepsilon * \delta)} \text{ (Ec. 1)}$$

Where:

A_{MAX} = Spectrophotometric Absorption at the wavelength of 450 nm.

M = Molecular mass of ß-carotene = 537 $g.mol^{-1}$.

ε = Molar extinction coefficient of ß-carotene in acetone (140663 $L.mol^{-1}.cm^{-1}$).

δ = Optical path (cm).

DISCUSSION

The wavelength plays a significant role in this method's accuracy. For instance, if the method is used to determine carotenoids, chlorophyll a, and chlorophyll b in leaves (Lichtenthaler et al., 2005), these authors suggested another equation:

$$C_{B-carotenoids} = \frac{(1000A_{470} - 1{,}82C_a - 85{,}02\ C_b)}{198} \text{ (Ec.2)}$$

Where A_{470} is absorption determined at the wavelength of 450 nm in UV-Vis spectrophotometer; C_a is α -chlorophyll concentration; and C_b is β -chlorophyll concentration. But if the purpose is to calculate only carotenoids equation (1) should be used.

Another critical factor to consider is the concentration of the solvent used (acetone), which can be 80% or 100% (v/v) depending on the biomass treatment. The speed and time of the centrifugation are also crucial variables affecting the carotenoid extraction yield, with various studies proposing different values (Corzo Piñeros et al., 2019; Lichtenthaler et al., 2005).

CONCLUSION

The spectrophotometric method for extracting and quantifying beta-carotenes from *Chlorella vulgaris* biomass is an essential process, providing valuable insights into their concentration and potential applications in various industries. Understanding the factors that affect the accuracy of determination will help researchers optimize the extraction procedure and obtain reliable results.

ACKNOWLEDGMENTS

To the Semillero Procycles, from Universidad de América for its constant support.

REFERENCES

Amrutkar, M. P. R., Badgujar, M. N. D., Bhamre, M. R. D., Chaudhari, M. V. A., & Borikar, M. S. P. (2020). Formulation and evaluation of photo-aging face gel by using algae powder extract (*Chlorella vulgaris*). *International Journal Of All Research Writings*, 2(12), 17-24.

Biehler E, Mayer F, Hoffmann L, Krause E, Bohn T (2010) Comparison of spectrophotometric methods for carotenoid determination in frequently consumed fruits and vegetables. *Journal of food science* 75 (1):C55-61. doi:10.1111/j.1750-3841.2009.01417.

Corzo Piñeros, R. J., Manrique Ruíz, I. G., Sandoval Herrera, J. A. y Rubio Fernández, D. (2019). Evaluación de carotenoides y lípidos en la microalga *Scenedesmus dimorphus* a escala laboratorio. *Mutis*, 9(1), 20-28, doi: 10.21789/22561498.1471

Del Campo, J. A., Moreno, J., Rodríguez, H., Vargas, M. A., Rivas, J., & Guerrero, M. G. (2000). Carotenoid content of chlorophycean microalgae: factors determining lutein accumulation in *Muriellopsis* sp. (Chlorophyta). *Journal of Biotechnology*, 76(1), 51-59.

Gebregziabher, B.S.; Zhang, S.; Qi, J.; Azam, M.; Ghosh, S.; Feng, Y.; Huai, Y.; Li, J.; Li, B.; Sun, J. Simultaneous Determination of Carotenoids and Chlorophylls by the HPLC-UV-VIS Method in Soybean Seeds. *Agronomy* 2021, 11, 758. https://doi.org/10.3390/agronomy11040758

Ibáñez, F. (2018). Protocolo para la determinación de carotenoides en frutos nativos. En: *Protocolos estandarizados por el PROCISUR para la Valorización de frutos nativos*. Pino, M. T., & Díaz, E. D. Editores Técnicos.

Lichtenthaler HK, Buschmann C (2001) Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. In: *Current Protocols in Food Analytical Chemistry*. John Wiley & Sons, Inc. doi:10.1002/0471142913.faf0403s01

COLORIMETRIC DETERMINATION OF ANIONIC SURFACTANTS BY MBAS METHOD IN SURFACE WATERS AND WASTEWATER

Sánchez Novoa, J.G[1], Rodríguez, N.[2], de Cabo, L.[2], Marconi, P.L.[1]
[1] Maimónides University-CEBBAD-CONICET. Buenos Aires, Argentina.
[2] Natural Science Argentine Museum B. Rivadavia-CONICET. Buenos Aires, Argentina.
Correspondence: juangabrielsancheznovoa@gmail.com

SUMMARY

This method is suitable for the determination of anionic surfactants in surface waters and wastewater. Anionic surfactants can be detected using a simple and precise method based on their activity with methylene blue active substance (MBAS). This method, derived and modified from IDEAM (2007), involves three consecutive extractions from an acidic aqueous solution, where an excess of methylene blue is added, into a chloroform organic phase. Subsequently, the blue color in the organic phase is measured by spectrophotometry at 652 nm (Standard Methods, 2005). However, it is important to consider the potential presence of other active substances that may react with methylene blue in the sample. Under controlled laboratory conditions, this methodology enables the precise quantification of MBAS concentrations commencing at 0.50mg/L.

INTRODUCTION

The discharge of untreated domestic and industrial wastewater into aquatic ecosystems presents a significant peril to water resources, biodiversity, and ecosystem services. Hence, it is imperative to investigate alternative techniques for wastewater treatment, including microalgae phytoremediation, which involves the removal or biotransformation of pollutants, such as nutrients and xenobiotics (Mishraa & Mohantya, 2019). The implementation of native microalgae strains in these systems may provide greater efficiency owing to their innate capacity to adapt to local conditions, resulting in better growth in untreated wastewater (Wen et al., 2017). However, given the susceptibility of microalgae to various pollutants, it is crucial to study and establish the limitations of these bioremediation approaches.

REAGENTS

- Distilled water for the preparation of all reagents and dilutions.

- Standard solution of sodium dodecyl sulfate (1000 mg/L), expressed as 1000 mg MBAS/L. The sodium salt of dodecyl sulfate ($C_{12}H_{25}NaO_4S$, MW = 288.38 g) with a purity of 96% was employed. Dissolve 1.042 g of sodium dodecyl sulfate salt in 500 mL of distilled water and dilute to 1000 mL. Store the solution refrigerated.
- Working solution of 10 mg/L of sodium dodecyl sulfate. Take 10 mL from the 1000 mg MBAS/L stock solution and transfer it to a class A 1000 mL volumetric flask. Bring to volume. Prepare this solution daily. When preparing this solution from the 1000 mg MBAS/L stock solution, it is crucial to homogenize the stock solution by bringing it to 25 °C, as slight salt precipitation may occur at temperatures below the laboratory's working temperature.
- Alcoholic phenolphthalein indicator solution. Dissolve 80 mg of phenolphthalein in 100 mL of absolute methanol.
- 1N Sodium hydroxide solution. Dissolve 40 g of 100% sodium hydroxide in 1 liter of distilled water.
- Sulfuric acid, H_2SO_4, 1N and 6N.
- Chloroform, $CHCl_3$. CAUTION: Chloroform is toxic and a potential carcinogen. Take appropriate precautions to prevent inhalation and skin exposure (Always work in an organic vapor hood and use safety equipment).
- 0.1% Methylene blue solution: Dissolve 100 mg of methylene blue (Eastman No. P573 or equivalent) in 100 mL of distilled water.
- Methylene blue reagent: Transfer 30 mL of the 0.1% methylene blue solution to a 1000 mL flask. Add 500 mL of distilled water, 41 mL of 6N H_2SO_4, and 50 g of $NaH_2PO_4 \cdot H_2O$ previously dissolved in 200 mL of distilled water. Stir until dissolved, then bring to volume.
- Washing solution: Add 41 mL of 6N H_2SO_4 to 500 mL of distilled water in a 1000 mL flask. Add 50 g of $NaH_2PO_4 \cdot H_2O$. Stir until dissolved and dilute to 1000 mL
- Hydrogen peroxide, H_2O_2, 30%.

MATERIALS

- Spectrophotometer with a 1 cm light path, to be used at 652 nm.
- Rotary evaporator.
- Organic vapor hood.
- Separatory funnels, preferably 250 and 500 mL in size, equipped with TFE stoppers and keys.
- Small glass filtration funnels with long stems.
- Class A volumetric flasks of 100, 200, 250, 500 and 1000 mL.
- Funnel supports.

- Beakers of 50, 100, 250, and 500 mL.
- Pipettes of 1, 2, 3, 4, 5 and 10 mL.
- Class A graduated cylinders of 20, 25, 50, and 100 mL.
- Glass wool or glass fiber.

Ensure all equipment is washed using soap or detergent that does not contain active substances, rinse with a diluted solution of sulfuric acid, and thoroughly rinse with abundant distilled water.

PREPARATION OF THE CALIBRATION CURVE

Prepare a series of separatory funnels containing 5, 10, 20, and 30 mL of a working solution of sodium dodecyl sulfate (SDS) at a concentration of 10 mg MBAS/L. The final concentrations of these standards after the extraction process will be 0.5, 1.0, 2.0, and 3.0 mg MBAS/L, respectively. Instead of using the suggested linear alkylbenzene sulfonate in the Standard Methods (5540 C), utilize sodium dodecyl sulfate salt for the assays. Ensure that each funnel is supplemented with sufficient distilled water to reach an approximate volume of 100 mL, as specified in Table 1.

Table 1: Calibration curve for MBAS determination.

	Concentration	Distilled water	Standard solution MBAS	Final Volume
	mg MBAS/L	**(mL)**	**(mL)**	**(mL)**
Blank	0	100	0	
STD 1	0.5	95	5	
STD 2	1	90	10	100
STD 3	2	80	20	
STD 4	3	70	30	
STD 5	5	50	50	
STD CTRL 1*	0.5	95	5	
STD CTRL 2*	2.5	75	25	

*Control standard; MBAS standard solution 10 mg/L.

Process each standard and the blank following the described procedure (treat the samples uniformly) and construct a calibration curve by plotting the absorbance against mg MBAS/L of sodium salt.

ANALYSIS PROCEDURE

Sample Extraction

Pour the indicated volume of the standard or an appropriate volume of the sample as follows: 100 mL if the expected MBAS concentration is below 5.0 mg MBAS/L, or an adequate volume to achieve a concentration between 0.5 and 5.0 mg MBAS/L. Add the necessary volume of distilled water to reach a total volume of 100 mL in a separatory funnel of either 250 mL or 500 mL capacity, as appropriate.

To each sample or standard, add 3 drops of phenolphthalein indicator, followed by a dropwise addition of 1N NaOH until a persistent pink color is obtained. Then, add 1N H_2SO_4 dropwise until the pink color disappears.



Figure 1: Neutralization (Standards with phenolphthalein indicator).

To prevent decolorization of methylene blue by sulfides, add three drops of 30% H_2O_2. Agitate the funnel and add 25 mL of methylene blue reagent along with 25 mL of chloroform. Agitate the funnel vigorously for 30 seconds, venting after each extraction, and let the phases separate. Excessive agitation can lead to emulsion formation. In case of persistent emulsions, add a small volume of isopropanol (<10 mL); the same volume of isopropanol should be added to all standards and the blank. Typically, calibration curves do not exhibit emulsion formation. Note that some samples may require a longer phase separation time than others. Before draining the chloroform layer, gently swirl and allow it to settle.



Figure 2: Standards with Methylene Blue and Chloroform (Phase separation for extraction).

Transfer the chloroform layer by pouring it into a second separatory funnel or a 100 mL beaker. Rinse the stem of the first funnel with a small amount of chloroform. Repeat the extraction two more times using 25 mL of chloroform each time. If the blue color of the aqueous phase weakens or disappears, discard it and repeat with a smaller volume of the sample. Combine all the chloroform extracts in the second separatory funnel. Add 50 mL of washing solution and agitate vigorously for 30 seconds; no emulsions should form at this stage. Allow it to settle. Extract the chloroform layer through a small glass funnel with a narrow stem containing a cotton plug into a 100 mL Class A volumetric flask; the filtrate should be clear. Extract the washing solution once more with 25 mL of chloroform. Collect the chloroform extracts in the 100 mL flask, fill it up to the calibration mark with chloroform, and mix thoroughly.

A) B)



Figure 3: A) Chloroform extracts with washing solution. **B)** Calibration curve for measurement at 652 nm.

Spectrophotometric Measurement

Turn on the UV-Vis Spectrophotometer. Ensure that the 1 cm glass cell is perfectly clean. If you observe any blue stains, leave it in soapy water, wash it with 5% HCl, and rinse it thoroughly with distilled water.

To begin the photometric readings, place the reagent blank and label it as BLANK. Proceed with the control standards in ascending order, starting from the lowest concentration, and read the samples at 652 nm.

Data Processing and Calculation of Results

The spectrophotometer provides results in Absorbance. The concentration in mg MBAS/L is determined by interpolating the measured absorbance values of the sample using the calibration curve equation while considering the dilution factor of each sample. This calculation is carried out using the following formula:

$$y = m * x + b$$

$$x = \frac{(y - b)}{m} * (Fd)$$

x = Calculated Surfactant Concentration (mg MBAS /L).
y = (Absorbance at 652 nm) Reading taken by the spectrophotometer.
b = Y-intercept.
m = Slope of the calibration curve.
Fd = Dilution factor.

RESULTS

This research aimed to evaluate the detergent tolerance of a native strain of *Chlorella vulgaris*, both in suspension and immobilized in alginate beads, to assess its effectiveness and applicability in domestic wastewater treatment.

A unialgal culture of *Chlorella vulgaris* strain LMPA-40 was obtained from the culture collection belonging to the Natural Science Faculty of the National University of Patagonia San Juan Bosco (Biological Data National System, SNDB-173). This culture was maintained on Murashige Skoog (MS) synthetic culture medium supplemented with sucrose (3% w/v) and indoleacetic acid (1 mg/L) as a growth regulator (MS, Murashige and Skoog 1962) in Erlenmeyer of 250 mL containing 50 mL of culture media. Cultures were kept at 24 ± 2°C in a shaker at 100 rpm, with

mixotrophic conditions and a photoperiod of 16 h PAR (14,000k, 400 µmol photon/ m2 /s). The experimental design involved *in vitro* culture of the microalgae and two treatments with commercially available detergents: (Det-A) for dishwashing and (Det-B) for laundry. Twenty-five and seventy-five µL of each detergent were tested by a supplement in 50 mL of synthetic wastewater (WS) (Hyungseok et al., 1999). *C. vulgaris* culture was used as inoculum to suspension cultures (20%) or immobilized in alginate beads (20%) in Erlenmeyer flasks following the methodology described in Marconi et al. (2022). The cultures were maintained as described above. Six replicates were employed for each treatment.

The concentration of anionic surfactants in each volume was determined using the protocol for MBAS determination in wastewater, resulting in 55.6 and 143.1 mg/L for (Det-A), and 13.7 and 44.2 mg/L for (Det-B). MS and WS media were used as controls.

After three days of treatment, biomass production was estimated through Neubauer chamber counting, dry weight, chlorophyll content, also, growth rate (µ), and doubling time (dt) were estimated as described in Sanchez Novoa (2020).

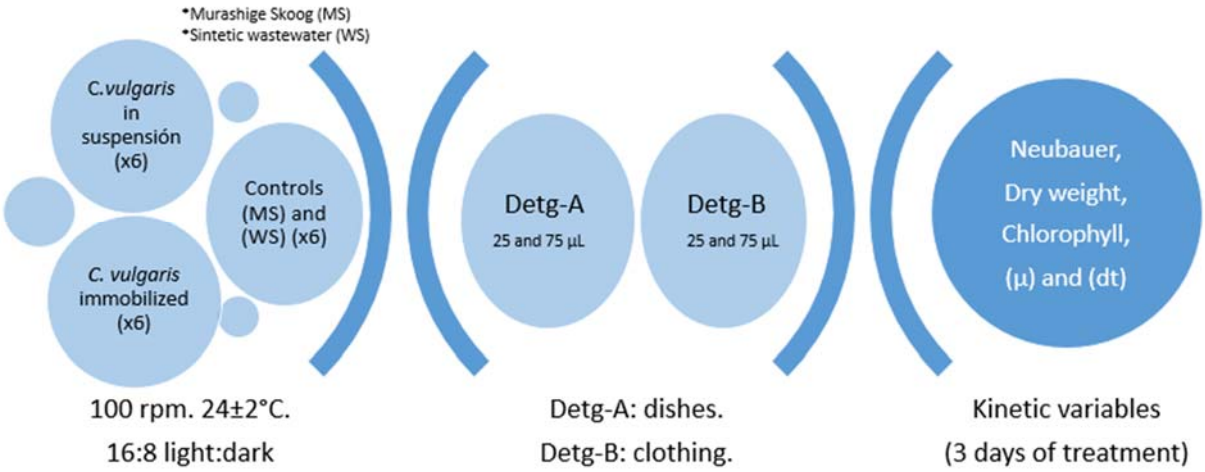


Figure 4: Methodological process diagram cultivation of *Chlorella vulgaris* with detergents.

The results indicated that algal growth was supported in (WS), except when treated with Det-A, which exhibited a toxic effect resulting in cell death (Sánchez-Novoa et al., 2023).

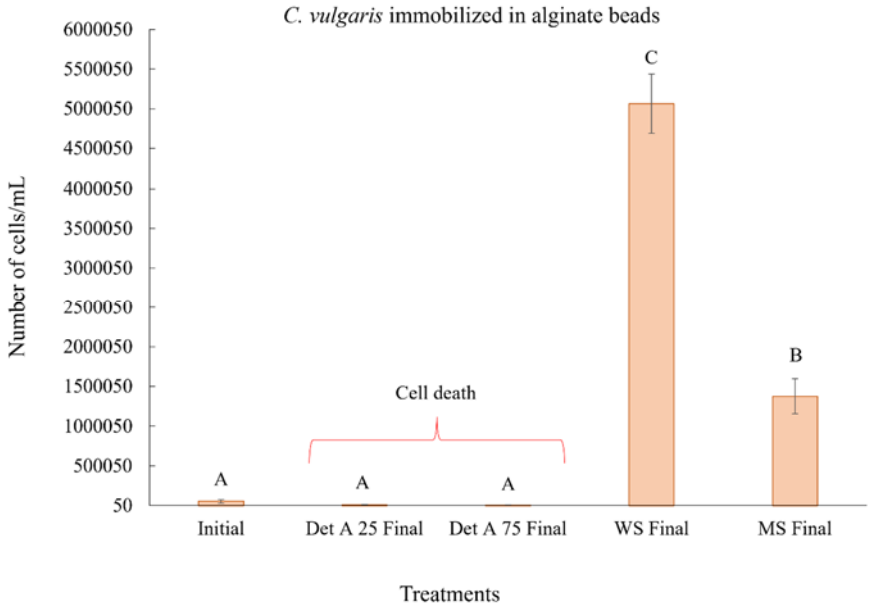


Figure 5: Biomass production of immobilized *Chlorella vulgaris* in alginate beads with (Det-A).

Comparing immobilized systems to suspension, it was observed that *C. vulgaris* demonstrated higher tolerance to Det-B in the first one. Immobilization in beads proved to be the most effective system when microalgae were exposed to detergents, achieving a dt of 0.65±0.06 and 3.02±1.7 days, and μ of 1.08±0.10 and 0.28±0.12 for low and high concentrations of Det-B, respectively. The immobilized system showed an increase in biomass production at low concentrations (2.96×10^6) as depicted in Figure 6. Additionally, there was an increase in chlorophyll content, although it was lower compared to the MS and WS controls.

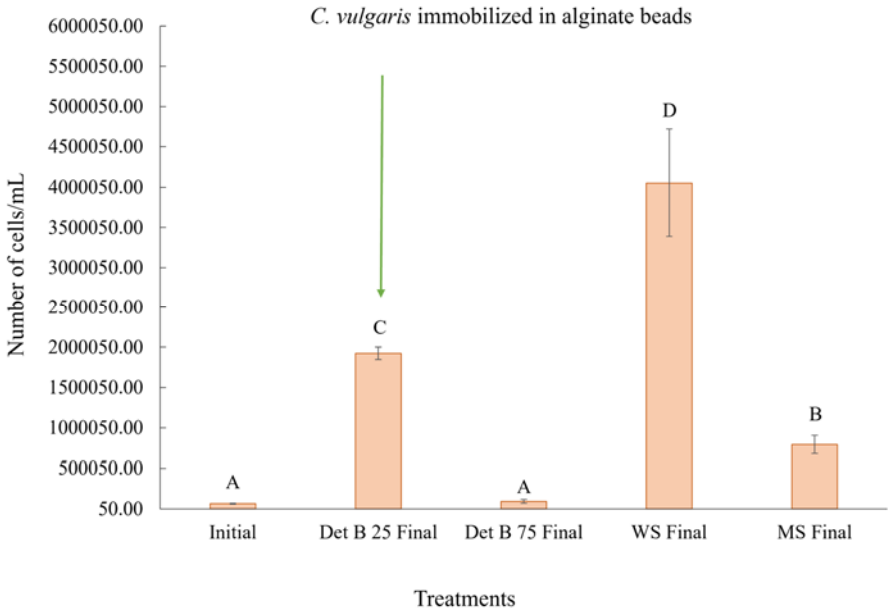


Figure 6: Biomass production of immobilized *C. vulgaris* in alginate beads with (Det-B).

In the suspension treatment, Det-B exhibited a dt of 5.20±0.95 and 10.38±1.27 days, and μ of 0.11±0.06 and 0.04±0.11 for low and high concentrations, respectively. The biomass production was (2.92 x 10^5) as illustrated in Figure 7.

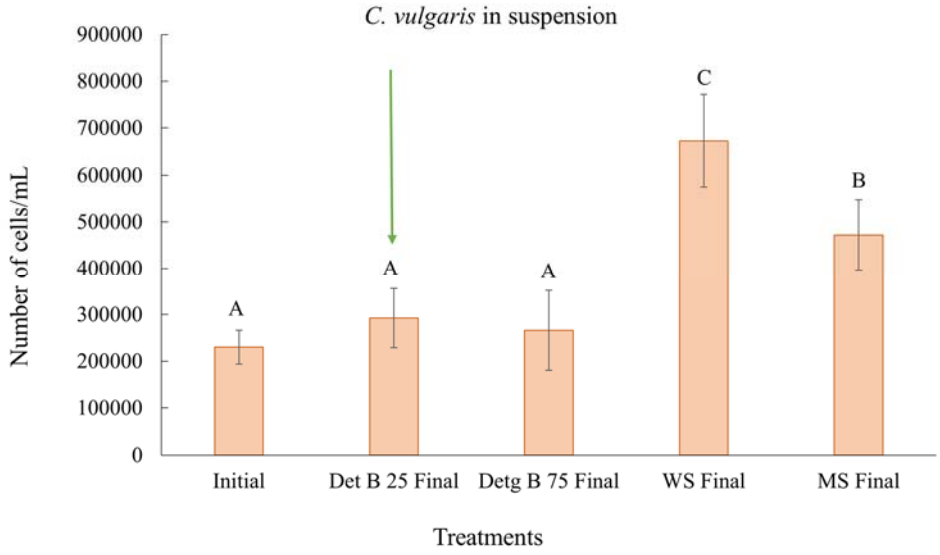


Figure 7: Biomass production of *Chlorella vulgaris* in suspension with (Det-B).

The tolerance of *C. vulgaris* to anionic surfactant substances decreases with increasing concentrations, with Detg-A reaching toxic levels beyond 50 mg/L. Nevertheless, the microalgae-based wastewater treatment system remains viable for wastewater containing MBAS levels higher than the guideline values established by the different national (Res. 79.179/90 and Law 26.221; < 5 mg/L) and provincial (ACUMAR: Res. 46/17 and ADA Res. 336/03; < 10 mg/L) that regulate the industrial and commercial discharges of liquid effluents in Argentina.

In conclusion, the results demonstrate a negative impact on microalgal biomass production when exposed to detergents in comparison to treatments without detergents.

REFERENCES

Hyungseok, Y., Kyu-Hong, A., Hyung-Jib, L., Kwang-Hwan, L., Youn-Jung, K., & Kyung-Guen, S. (1999). nitrogen removal from synthetic wastewater by simultaneous nitrification and denitrification (SND) via nitrite in an intermittently-aerated reactor. Pergamon,, 33, 145-154.

IDEAM. (2007). Tensoactivos aniónicos en agua – MÉTODO SAAM. Instituto de Hidrología, Meteorología y Estudios Ambientales. Ministerio de Ambiente, Vivienda y Desarrollo Territorial – República de Colombia.

Marconi, P.L., Zawoznik, M, Groppa, M.D., deCabo, L.I. Chapter 2: Inmovilización de microalgas en perlas de alginato para su uso en procesos de biorremediación. Ed. Navarro Llorens, J. M., & Gouveia, L. (2022). Protocolos de microalgas de la Red RENUWAL.

Mishraa, S., & Mohantya, K. (2019). Comprehensive characterization of microalgal isolates and lipid-extracted biomass as zero-waste bioenergy feedstock: An integrated bioremediation and biorefinery approach. Bioresource Technology, 273, 177-184. doi:https://doi.org/10.1016/j.biortech.2018.11.012

Sánchez-Novoa, J., Marconi, P., & de Cabo, L. (2023). XIV SIMPOSIO- 2023, REDBIO ARGENTINA, "BIOTECNOLOGÍA PARA UN MUNDO EN CAMBIO". Ficorremediación utilizando cultivos de una cepa autóctona de Chlorella vulgaris. Buenos Aires., Argentina.

Wen, Y., He, Y., Ji, X., Li, S., Chen, L., Zhou, Y., Chen, B. (2017). Isolation of an indigenous *Chlorella vulgaris* from swine wastewater and characterization of its nutrient removal ability in undiluted sewage. Bioresource Technology, *243*, 247-253. doi:https://doi.org/10.1016/j.biortech.2017.06.094

Standard Methods for the Examination of Water and Wastewater (2005). American Public Health Association, American Water Works Association, Water Pollution Control Federation. 21ed., New York.

DETERMINATION OF PROTEINS IN MICROALGAL BIOMASS

Trentini, A.[1], Rodríguez, N.[2], Sánchez Novoa, J.G.[1], de Cabo, L.[2], Marconi, P.L.[1]

[1] Maimonides University-CEBBAD-CONICET. Buenos Aires, Argentina.

[2] Museo Argentino de Ciencias Naturales B. Rivadavia-CONICET. Buenos Aires, Argentina.

Correspondence: andretrentini.89@gmail.com

SUMMARY

Several colorimetric techniques are available for the quantitative measurement of proteins; among these, the most frequently employed methods are those developed by Bradford (1976) and Lowry et al. (1951). The Lowry method is based on the production of chemical species that are absorbed at a specific wavelength in proportion to the amount of analyte present in the sample. Its advantage lies in its sensitivity, which is around 10 μg/mL.

INTRODUCTION

Microalgae are photosynthetic microorganisms that can effectively purify polluted water, offering advantages over traditional methods. They can produce biomass rich in a wide variety of bioproducts such as proteins, among other bioactive compounds (Braeken et al., 2004; Doubla et al., 2007; Cai et al., 2013; Batista et al., 2015; Singh et al., 2019; Agüera et al., 2020; Gonzalez-Lopez et al., 2020). Total protein (TP) quantification is achieved through methodologies based on the production of chemical species that absorb light at a given wavelength in proportion to the amount of the analyte present in the sample. In particular, the method of Lowry et al. (1951) has the advantage of being extremely sensitive, being capable of detecting quantities on the order of 10 μg TP/mL. The main drawback of the method arises when assessing phenols present in the protein (primarily tyrosine residues). The intensity of the resulting color varies between different proteins. To minimize this, when dealing with intricate biological mixtures, the method is calibrated using a commercial protein, such as bovine serum albumin (BSA).

The reaction involved in the Lowry method is quite complex and proceeds in the following steps:

Reaction Mechanism for Protein Quantification

1. Pre-reaction of the protein in an alkaline medium with Cu^{2+} ions, in the presence of tartrate to avoid precipitation. This process is identical to the Biuret reaction, and it results in the formation of a coordination complex between copper and peptidic nitrogen.
2. Reaction follows with the application of the Folin-Ciocalteau reagent, known for its response to phenolic compounds (phosphomolybdotungstic acid). This reagent is reduced through the phenolic groups (and to a lesser extent, imidazole, and indole groups) present in the protein. This reduction forms a dark blue complex, which is quantified colorimetrically at 750 nm. The choice between the two reactions depends on the protein concentration of the sample under study.

MATERIALS

- Spectrophotometer
- Centrifuge
- Vortex mixer
- Thermostatic bath
- 2 mL and 5 mL Eppendorf tubes
- Micropipettes of 10 µL, 100 µL, 200 µL, and 1000 µL
- Pipettes of 1 mL, 2 mL, 5 mL, and 10 mL
- Test tubes or hemolysis tubes
- Precipitation beakers of 50 mL, 100 mL, 250 mL, and 500 mL
- Class A volumetric flasks of 100 mL and 200 mL

All glassware should be pre-washed with 0.1% HCl and rinsed three times with distilled water. Allow to air dry before use.

LOWRY METHOD:

Preparation of Reagents and Lowry Calibration Curves

Preparation of Lowry´s Reagent

- **2% Sodium Carbonate Solution (w/v):** Weigh 2 g of Sodium Carbonate, add 0.4 g of NaOH, and dissolve in distilled water to make a final volume of 100 mL.

- **1% Copper Sulfate Solution (w/v):** Dissolve 1 g of $CuSO_4 \cdot 5H_2O$ in 100 mL of distilled water.
- **2% Potassium-Sodium Tartrate Solution (w/v):** Dissolve 2 g of $KNaC_4H_4O_6 \cdot 4H_2O$ in 100 mL of distilled water.
- **Mix, at the time of use, in the following proportions:** 50 mL of 2% Na_2CO_3 + 0.5 mL of 1% $CuSO_4 \cdot 5H_2O$ + 0.5 mL of 2% $KNaC_4H_4O_6 \cdot 4H_2O$.
- **Folin-Ciocalteau Reagent (1:1):** Prepare a solution of Folin-Ciocalteau reagent in a 1:1 ratio diluted in distilled water.

Preparation of Calibration Curve

The calibration curve is prepared in duplicate or triplicate using pre-treated test tubes.

- 1 mg/mL Bovine Serum Albumin (BSA) Solution: Dissolve 1 mg of BSA in 1 mL of distilled water.
- 1N Sodium Hydroxide Solution: Dissolve 40 g of 100% Sodium Hydroxide in 1 L of distilled water.
- Label the tubes from 1 to 7.
- Pipette the microliters of standard solution as indicated in column 2 of Table 1 into each tube.
- Add microliters of water according to the indications in column 4 of the table.
- Add 5 mL of Lowry Reagent to each tube.
- Shake vigorously in Vortex for 30 seconds. Let the tubes rest for 15 minutes.
- Add 500 µL of Folin-Ciocalteau Reagent (1:1).
- Shake vigorously in Vortex for 30 seconds. Let the tubes rest for 30 minutes.
- Measure the absorbance at 750 nm in a spectrophotometer.

Table 1: Calibration curve for protein determination using Lowry Method

Tube	std. BSA (µL)	BSA (mg/mL)	H_2O (µL)	Lowry Reagent (mL)
1	0	0	1000	5
2	50	0.05	950	5
3	100	0.10	900	5
4	150	0.15	850	5
5	200	0.20	800	5
6	300	0.30	700	5
7	400	0.40	600	5

Sample Preparation

The samples must come from microalgal biomass extracted according to the steps indicated below:

1. Centrifuge a culture sample of *C. vulgaris* for 15 minutes at 15,000 g, coming from the bioremediation process, until obtaining 0.3 g of fresh weight biomass.
2. Hydrolyze the biomass by adding 1.5 mL of 1 M NaOH.
3. Heat the mixture in a thermostatic water bath at 95°C for 1 hour.
4. Centrifuge at 15,000 g for 15 minutes.
5. Transfer 100 µL of the supernatant to a test tube and add 900 µl of distilled water.
6. Add 5 mL of Lowry Reagent to each tube.
7. Vortex vigorously for 30 seconds. Let the test rest for 15 minutes.
8. Add 500 µL of Folin-Ciocalteau Reagent (1:1).
9. Vortex vigorously for 30 seconds. Let the tubes rest for 30 minutes.
10. Measure the absorbance at 750 nm in a spectrophotometer.

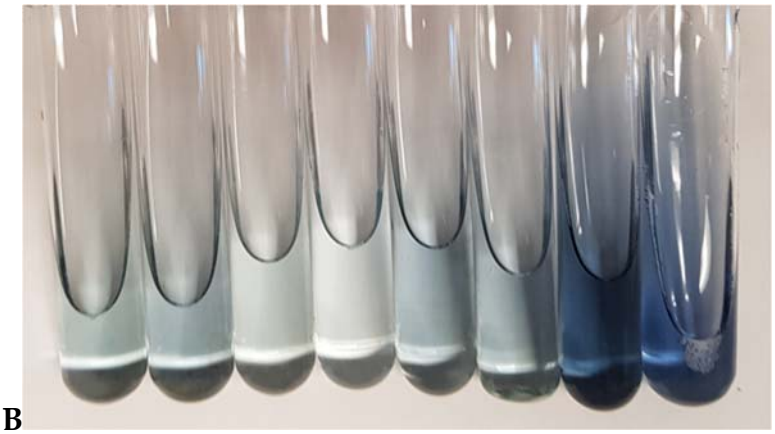
A  **B** 

Figure 1: Calibration curve (Lowry Method) (A); Extracts of *C. vulgaris* after water treatment at Ao. Cildañez (B).

Sample Reading

To begin with, photometric readings, start with the reagent blank and label it as "BLANK." Then proceed with the control standards in ascending order, starting from the lowest concentration, and read the samples in the 750 nm wavelength range.

Data Processing and Result Calculation

By comparing the obtained readings with the calibration curve, protein concentration is obtained and expressed in mg/mL. Dry biomass measurement of a culture sample is taken at the time of the assay to express protein concentration as a mass percentage of dry weight (% DW).

RESULTS

Bioremediation using Immobilized Chlorella Cultures

In previous studies, it was observed that immobilized *Chlorella vulgaris* cultures growing within alginate beads for 5 days in water samples from an urban stream (Cildañez stream) were able to decrease the concentration of several contaminants (Groppa et al., 2019; Marconi et al., 2020). Protein concentration could be used as an indicator of microalgae growth and metabolism.

Water samples

Three water samples randomly obtained were collected in the spring of 2018 from Cildáñez stream (34°67′60.00″S; 58°44′37.06″W) and analyzed in the laboratory of APRA (Agencia de Protección Ambiental, Ciudad Autónoma de Buenos Aires).

Experimental design

A unialgal culture of *Chlorella vulgaris* strain LMPA-40 (Biological Data National System, SNDB-173) was maintained on Murashige Skoog (MS) synthetic culture medium supplemented with sucrose (3% w/v) and indoleacetic acid (1 mg/L) as a growth regulator (Murashige and Skoog, 1962) in Erlenmeyer of 250 mL containing 50 mL of culture media. The immobilization technique was described in Marconi et al. (2022). Cultures were kept at 24 ± 2°C in a shaker at 100 rpm, with mixotrophic conditions and photoperiod of 16 h (14,000k, 400 μmol photon/ m^2 /s).

Stirred-tank bioreactors (Minifors, Infors HT®, Switzerland) with a working volume of 1.5 L feed with Cildáñez residual water (Cild) or MS synthetic culture medium were used. The bioreactor was inoculated with 2% (w/v) *C. vulgaris* immobilized in alginate (100 beads). The immobilization process is described in Marconi et al. (2022). The bioreactor includes mechanical agitation achieved by a marine Rushton propeller (100 rpm), a porous metal sparger to supply bubble aeration (0.5 vvm), and a photoperiod of 16 h PAR.

The growth of *C. vulgaris* along the culture period was assessed by dry weight (DW) and counting algae cells in a Neubauer chamber after dissolving the alginate matrix, as previously described (Trentini et al. 2017; Marconi et al., 2022).

Experiments and analytical determinations were performed in triplicate. The results were evaluated by ANOVA with post-hoc Tukey test for multiple comparisons using Infostat software (Tukey 1953).

RESULTS

Experiments were carried out to assess the effect of residual water from the Cildáñez stream on the primary metabolism of *C. vulgaris* immobilized in alginate. For this purpose, the total protein (TotalP) content in the biomass after each bioprocessing was studied.

Upon analysis of the TotalP percentage, it was observed that it varied between 10% and 40.45% among the biomass of immobilized microalgae after each bioprocess with Cild residual water (Fig. 2). Therefore, the distribution of the obtained findings seems to be spread, which can be attributed to the random selection of water samples. These observations were previously described when nitrate and phosphorus were studied (Groppa et al., 2019).

Finally, the bioprocess conducted in MS medium yielded the highest protein concentration of 55.9% (same figure).

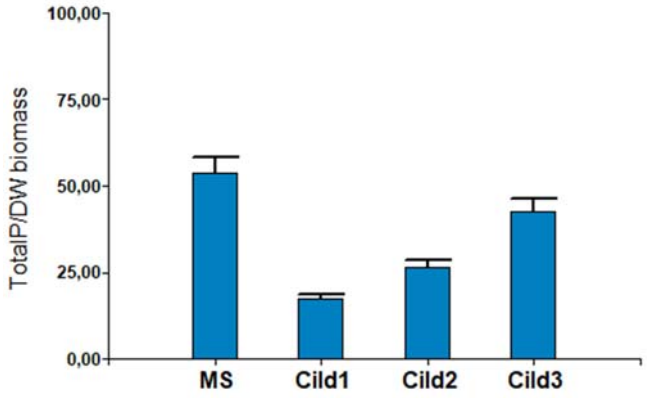


Figure 2: Total Protein content expressed as a percentage of biomass (% TotalP/DW biomass) from bioprocesses conducted in control medium (MS) and Ao. Cildáñez residual water samples (Cild 1-3) in *C. vulgaris* cultures. Letters denote groupings with statistically significant differences based on the ANOVA test followed by Tukey's HSD test.

In conclusion, it can be stated that the Lowry methodology for quantifying TotalP in microalgal biomass is both easy and fast.

REFERENCES

Agüera A, Plaza-Bolaños P, & Fernández FA. 2020. Removal of contaminants of emerging concern by microalgae-based wastewater treatments and related analytical techniques. Current Developments in Biotechnology and Bioengineering, 1(1):503-525. doi: 10.1016/B978-0-12-819594-9.00020-6.

Batista AP, Ambrosano L, Graça S, Sousa C, Marques PA, Ribeiro B, Botrel EP, Castro-Neto P & Gouveia, L. 2015. Combining urban wastewater treatment with biohydrogen production–an integrated microalgae-based approach. Bioresource Technology, 184(1):230-235. doi: 10.1016/j.biortech.2014.10.064

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Braeken L, Van der Bruggen B, Vandecasteele C. Regeneration of brewery waste water using nanofiltration. Water Res. 2004;38(13):3075-3082. doi:10.1016/j.watres.2004.03.028.

Cai T, Park SY, Li Y. 2013. Nutrient recovery from wastewater streams by microalgae: status and prospects. Renew Sustain Energy Rev, 19(1):360-369. doi: 10.1016/j.rser.2012.11.030.

Doubla A, Laminsi S, Nzali S, Njoyim E, Kamsu-Kom J, Brisset JL. 2007. Organic pollutants abatement and biodecontamination of brewery effluents by a non-thermal quenched plasma at atmospheric pressure. Chemosphere, 69(2):332-337. doi: 10.1016/j.chemosphere.2007.04.007

Groppa, M. D., Trentini, A. G., Zawoznik, M. S., Bigi, R. I., Nadra, C., & Marconi, P. L. (2019). Optimization of a bioremediation strategy for an urban stream of Matanza-Riachuelo basin. International Journal of Environmental and Ecological Engineering 13: 418-424

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–275.

Marconi, P. L., Trentini, A., Zawoznik, M., Nadra, C., Mercadé, J. M., Sánchez Novoa, J. G., ... & Groppa, M. D. (2020). Development and testing of a 3D-printable polylactic acid device to optimize a water bioremediation process. AMB Express, 10(1), 1-9.

Marconi, P.L., Zawoznik, M, Groppa, M.D., deCabo, L.I. Chapter 2: Inmovilización de microalgas en perlas de alginato para su uso en procesos de biorremediación. Ed. Navarro Llorens, J. M., & Gouveia, L. (2022). Protocolos de microalgas de la Red RENUWAL.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plantarum, 15: 473-497.

Singh J, Dhar DW. 2019. Overview of carbon capture technology: microalgal biorefinery concept and state-of-the-art. Front Mar Sci. 1(1):6:29. doi: 10.3389/fmars.2019.00029.

Trentini, A., Groppa, M. D., Zawoznik, M., Bigi, R., Perelman, P. E., & Marconi, P. M. (2017). Biorremediación del lago Lugano de la Ciudad Autónoma de Buenos Aires por algas unicelulares—estudios preliminares para su posterior utilización. Terra Mundus 4. Terra Mundus, 4.

Tukey J. W (1953) Some selected quick and easy methods of statistical analysis. Trans NY Acad. Sc., vol. 16: 88–97

OBTAINING PHOTOSYNTHESIS RATE MODELS AS A FUNCTION OF LIGHT USING PHOTO-RESPIROMETRY

Ana Sánchez Zurano[1,2], Silvia Villaró Cos[1,2], José María Fernández Sevilla[1,2], Francisco Gabriel Acién Fernández[1,2]

[1] Department of Chemical Engineering, Universidad de Almería, E04120 Almería, Spain

[2] Solar Energy Research Centre (CIESOL), Joint Centre University of Almería-CIEMAT, Almería, 04120, Spain

SUMMARY

The growth of microalgae depends on variables such as pH, temperature, nutrient concentration, dissolved oxygen, and the light they are exposed to. Among all these factors, the primary limiting factor and the most challenging to control is light. Therefore, evaluating the influence of light on microalgae cultivation is essential as a first step to optimize the productivity of any system. In this protocol, the use of photo-respirometric techniques is proposed to determine the impact of available light quickly and economically on the net photosynthesis rate of the cultures. The proposed methodology allows quantifying the net photosynthesis rate within a light range of 0 y 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$, based on measurements of dissolved oxygen concentration variation under certain conditions. The obtained experimental values were fitted to a model to determine its characteristic parameters for each culture. Using this methodology enables the characterization of microalgae cultures in a simple and rapid way, reducing the required time compared to other methodologies. As an example, the developed methodology has been employed to evaluate and model the influence of light on the photosynthesis rate of three industrially relevant strains: *Scenedesmus almeriensis*, *Chlorella vulgaris,* and *Nannochloropsis gaditana*.

INTRODUCTION

Microalgae are photosynthetic organisms with simple nutritional requirements that enable their large-scale growth using light as an energy source and CO_2 as a carbon source. Their rapid and cost-effective growth allows microalgae biomass to be harnessed for the development of products such as biostimulants, biopesticides, animal feed, cosmetics, or functional foods for human nutrition (Nagarajan et al. 2021). However, for their large-scale industrial production, the variables or parameters that determine their growth must be optimized. Among these parameters are light, pH, temperature, dissolved oxygen, and nutrient concentration. Currently, in large-scale microalgae production systems, it is possible to control the pH through on-demand

injection of CO_2, dissolved oxygen through air injection, and nutrient concentration (especially nitrogen and phosphorus) through the input medium (Pawlowski et al. 2016). Regarding temperature, when working with large-scale photobioreactors, temperature control becomes complex and expensive, making it feasible only for high-value applications. For large-scale processes, various alternatives have been proposed for temperature control, such as adjusting the culture depth or utilizing geothermal energy (Rodríguez-Miranda et al. 2022). Therefore, most of the variables that regulate microalgae growth can be controlled to operate under optimal or near-optimal conditions. However, large-scale microalgae production in outdoor settings is determined by light, which is a variable that cannot be controlled and depends on the season and location of the production systems (Solimeno et al. 2015).

Hence, microalgae production in outdoor conditions implies that light availability is determined by solar radiation at the reactor's surface, which is influenced by the reactor's location and geometry. It's important to note that this availability continuously changes based on the time of day and season of the year. Consequently, light availability becomes the limiting factor for maximizing production since nutrients and other parameters can be controlled. Therefore, evaluating the influence of light on the cultures' activity is crucial for production optimization. Over decades, growth models based on light have been developed, evolving into valuable tools for optimizing the production of the desired strain (Acién et al. 2016; Shoener et al. 2019).

Before investigating the influence of light on microalgae growth, it is feasible to determine the impact of light on photosynthetic activity. Once the net oxygen production of a culture or net photosynthesis rate is known, the growth rate of the culture can be derived. This approach makes it possible to avoid conducting multiple *batch* growth experiments to assess the influence of light on microalgae cultures. These experiments involve lengthy waiting times, ranging from weeks to months, and entail significant economic costs (Sánchez Zurano et al. 2021).

To mitigate the time and cost associated with these lengthy experiments, the practice of conducting photo-respirometric experiments is becoming more widespread. In these experiments, the net photosynthesis rate of the strain of interest is determined under specific light conditions. By assessing oxygen production, the specific growth rate can be estimated. As a result, these methodologies offer an improvement over conventional methods, as they provide a more efficient way to study and understand growth behaviors (Tang et al. 2014; Sforza et al. 2019). The use of photo-respirometric techniques based on oxygen production and consumption is rapid and easily attainable. These techniques facilitate the acquisition of growth models based on light dependency (Rossi et al. 2020).

This protocol introduces the application of photo-respirometric techniques to investigate the impact of light on oxygen production and consumption in three strains of microalgae. Additionally, it includes the subsequent modeling of the experimental data using a photosynthesis rate model based on light. Specifically, as an illustrative example, it demonstrates the modeling of light influence on three strains of industrial interest: *Scenedesmus almeriensis*, *Chlorella vulgaris,* and *Nannochloropsis gaditana.* The experimental data obtained through photo-respirometry were fitted to the Molina model (Grima et al. 1994), and characteristic parameters for each strain were derived.

REACTIVE

A designed apparatus is available to determine variations in dissolved oxygen concentration under controlled conditions. These variations allow for the determination of the Net Photosynthesis Rate (NPR) of the culture for each tested condition. This device comprises the following components (Figure 1):

- Flat-bottomed beaker with a thermostatic jacket, with a volume of 100 mL.
- Magnetic stirring system to maintain constant agitation of the culture within the beaker.
- LED lamps on both sides of the beaker, which can be regulated to provide the desired average irradiance within the culture.
- Computer with DaqFactory software to regulate measurement conditions and record data.
- Diffuser to supply air to regulate the initial dissolved oxygen concentration for measurements. A CO2 diffuser is also included to regulate pH.
- Sensors for measuring irradiance (QSL-1000 model, Walz), temperature (PT-100 model), pH (Hamilton 242428-4313 model), and dissolved oxygen (Hamilton 243650 model). These sensors are immersed in the culture within the beaker.
- Temperature maintenance is achieved through a thermoregulated bath connected to the beaker.

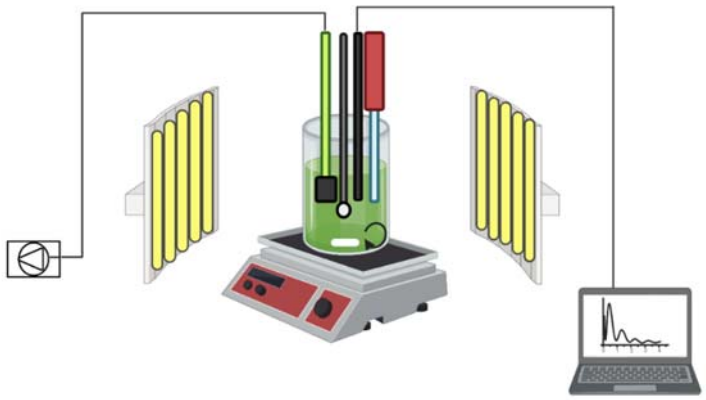


Figure 1. Scheme of photo-respirometer.

PROTOCOL

To obtain net photosynthesis rate (NPR) values at different irradiances, you start with an 80 mL sample of the culture. Here is the process:

1. The culture is placed in the jacketed vessel of the respirometer, ensuring that the light sensor is positioned in the center of the culture.
2. Other sensors are positioned above the light sensor to prevent creating shaded areas.
3. The culture must be constantly agitated to prevent sedimentation.
4. Oxygen Production Rate (OPR) will be determined during the light period, and Oxygen Consumption Rate (OCR) during the dark period.
5. Before starting measurements, the culture is aerated until reaching 100% oxygen saturation. Once the culture is at 100%, OPR and OCR determination can begin.
6. To determine OPR, the culture will be exposed to the desired irradiance for 4 minutes. The first minute is discarded as an adaptation period. The program calculates the slope of the increase, excluding the first minute, and provides a value in $mgO_2 \cdot L^{-1} \cdot h^{-1}$. To obtain OPR, this value should be normalized based on the biomass concentration (Equation 1).

$$OPR = \frac{1}{Cb} \cdot \left(\frac{d[DO]}{dt}\right) \qquad \text{Equation 1}$$

7. To determine OCR, the culture will be aerated again to reduce dissolved oxygen to 100% air saturation, and the culture will be kept in darkness for 4 minutes. The first minute is discarded due to adaptation time. The program

calculates the slope of the decrease, excluding the first minute, and provides a value in $mgO_2 \cdot L^{-1} \cdot h^{-1}$. To obtain OCR, this value should be normalized based on the biomass concentration (Equation 2).

$$OCR = \frac{1}{Cb} \cdot \left(\frac{d[DO]}{dt}\right) \quad \text{Equation 2}$$

8. Finally, the net photosynthesis rate (NPR) at each irradiance will be determined as the difference between OPR and OCR (Equation 3):

$$NPR = OPR + OCR \quad \text{Equation 3}$$

9. This protocol will be conducted in triplicate for each of the desired irradiances, starting from 0 up to 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$.
10. After obtaining the experimental NPR values at different average irradiances, the values will be fitted to a growth model using STATGRAPHICS Centurion. For this, the experimental data obtained at various irradiance levels should be input.
11. Next, a nonlinear regression is performed, relating the irradiance values as the independent variable and NPR values as the dependent variable.
12. Following this, the function to which the experimental data will be fitted must be entered. In this study, the equation proposed Grima et al. 1994., (Equation 4):

$$PO_2 = \frac{PO_{2max}\ x\ I^n}{I_k^n + I^n} \quad \text{Equation 4}$$

13. Once the equation is entered, it is necessary to provide approximate values for the parameters included in the model. In the example of this study, the Molina model (Grima et al. 1994) was used, which includes the net photosynthesis rate (PO_{2max}), the light affinity constant (I_k), and the shape parameter (n).
14. Finally, Statgraphics will find the best fit for the provided experimental values.

RESULTS

This methodology has been employed to model the influence of light on the photosynthesis rate of three strains of industrial interest: *Scenedesmus almeriensis*, *Chlorella vulgaris*, and *Nannochloropsis gaditana* at 30°C (Figure 2). An increase in NPR was observed as the available irradiance in the culture increased. In the case of *Nannochloropsis gaditana* culture, NPR increased exponentially up to 200 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$; beyond this value, NPR remained stable. For *Scenedesmus almeriensis* and *Chlorella*

vulgaris cultures, NPR increased up to 500 $\mu mol_{photons} \cdot m^{-2} \cdot s^{-1}$; beyond this irradiance value, NPR remained constant. At the tested temperature (30°C) and pH 8, the maximum experimentally obtained NPR was 297.22 ± 5.1 $mgO_2 \cdot g^{-1} \cdot h^{-1}$ for *Scenedesmus almeriensis*. For *Chlorella vulgaris*, the maximum NPR was 300.82 ± 4.9 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, while for *Nannochloropsis gaditana*, it was 191.89 ± 4.5 $mgO_2 \cdot g^{-1} \cdot h^{-1}$.

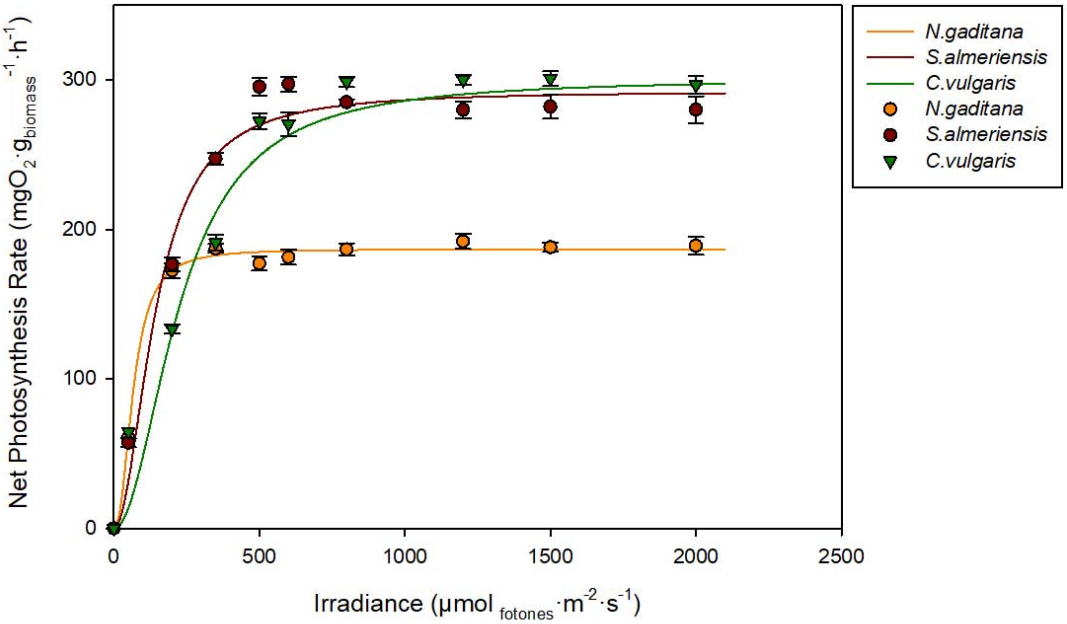


Figure 2. Influence of light on the net photosynthesis rate of *Scenedesmus almeriensis*, *Chlorella vulgaris*, and *Nannochloropsis gaditana*.

Once the experimental data was acquired, it was fitted to the photosynthesis rate model proposed by Grima et al. 1994 (originally a growth model), using STATGRAPHICS Centurion. The fitting of NPR data against average irradiance allowed us to extract the characteristic parameters of the model: maximum photosynthesis rate (PO_{2max}), light affinity constant (Ik), and shape parameter (n) (Table 1).

Table 1. Values of the parameters obtained when modeling the photosynthesis rate of *Scenedesmus almeriensis*, *Chlorella vulgaris*, and *Nannochloropsis gaditana.*

Parameters	*Scenedesmus almeriensis*	*Chlorella vulgaris*	*Nannochloropsis gaditana*
PO_{2max}	292	300	187
I_k	145	228	68
n	2	2	2

DISCUSSION

Currently, the most widely accepted method for optimizing the productivity of microalgae cultivation in an industrial-scale system involves exposing the strain of interest to different environmental and operational conditions to determine its maximum specific growth rate. However, these experiments require significant resources and lengthy waiting times. Therefore, the influence of environmental and operational parameters, such as light, can be evaluated in the laboratory by simulating external conditions. One of the methods with the greatest potential to achieve this goal is photo-respirometry (Sforza et al. 2019). This technique is based on determining the production and consumption of oxygen by the strain of interest under controlled conditions. The experimental values obtained allow for the calculation of the NPR at different light levels. (Yun and Park 2003; Ippoliti et al. 2016). Once the NPR values are obtained, by employing photosynthesis rate models, it is possible to determine the maximum photosynthesis rate for each strain under study, and subsequently relate it to the maximum growth rate (Sánchez-zurano et al. 2021). In this study, the influence of light on the cultivation of three microalgal strains, *Scenedesmus almeriensis*, *Chlorella vulgaris*, and *Nannochloropsis gaditana*, was assessed using photo-respirometry. Experimental results demonstrated that light significantly ($p<0.05$) affects the net photosynthesis rate of the studied strains. The experimental data were used to model the photosynthesis rate of each strain as a function of light, employing the model proposed by Grima et al. 1994. This model allows for the determination of the maximum photosynthesis rate (PO_{2max}) of the strain of interest under the tested conditions, while also calculating the initial slope (I_K), both of which are key parameters in the design of microalgae-based processes in photobioreactors. This same methodology can be employed to assess the influence of light during different seasons of the year, which is highly valuable for predicting system productivity (Rodríguez-Miranda et al. 2022). It can also be used to simulate the system's response in the event of a control failure that may lead to an inappropriate pH situation (Rossi et al. 2020). Therefore, the use of photo-respirometry along with mathematical models is a quick and valuable tool for characterizing strains of interest under selected conditions, saving time and resources compared to lengthy experiments.

ACKNOWLEDGMENTS

This research is part of the Horizon 2020 Research and Innovation Framework Program (project: PRODIGIO, 101007006). Additionally, this study has received support from the Campus of International Excellence of the Sea (CEIMAR) through the Young Research Projects Program 2022 (project: ALGABIOTIC) and the Ibero-American Network for Wastewater Treatment with Microalgae (RENUWAL).

REFERENCES

Acién FG, Gómez-Serrano C, Morales-Amaral MM, et al (2016) Wastewater treatment using microalgae: How realistic a contribution might it be to significant urban wastewater treatment? Appl. Microbiol. Biotechnol. 100:9013–9022

Grima EM, Camacho FG, Pérez JAS, et al (1994) A mathematical model of microalgal growth in light-limited chemostat culture. J Chem Technol Biotechnol 61:167–173. https://doi.org/10.1002/jctb.280610212

Ippoliti D, Gómez C, del Mar Morales-Amaral M, et al (2016) Modeling of photosynthesis and respiration rate for Isochrysis galbana (T-Iso) and its influence on the production of this strain. Bioresour Technol 203:71–79. https://doi.org/10.1016/j.biortech.2015.12.050

Nagarajan D, Varjani S, Lee DJ, Chang JS (2021) Sustainable aquaculture and animal feed from microalgae – Nutritive value and techno-functional components. Renew Sustain Energy Rev 150:. https://doi.org/10.1016/j.rser.2021.111549

Pawlowski A, Frenández I, Guzmán JL, et al (2016) Event-based selective control strategy for raceway reactor: A simulation study. IFAC-PapersOnLine 49:478–483. https://doi.org/10.1016/J.IFACOL.2016.07.388

Rodríguez-Miranda E, Sánchez-Zurano A, Guzmán JL, et al (2022) A seasonal simulation approach for culture depth influence on the temperature for different characterized microalgae strains. Biotechnol J 17:2100489. https://doi.org/10.1002/BIOT.202100489

Rossi S, Casagli F, Mantovani M, et al. (2020) Selection of photosynthesis and respiration models to assess the effect of environmental conditions on mixed microalgae consortia grown on wastewater. Bioresour Technol 305:122995. https://doi.org/10.1016/j.biortech.2020.122995

Sánchez-zurano A, Rodríguez-miranda E, Guzmán JL, et al (2021) ABACO: A New Model of Microalgae-Bacteria Consortia for Biological Treatment of Wastewaters. Appl Sci 2021, Vol 11, Page 998 11:998. https://doi.org/10.3390/APP11030998

Sánchez Zurano A, Gómez Serrano C, Acién-Fernández FG, et al. (2021) Modeling of photosynthesis and respiration rate for microalgae–bacteria consortia. Biotechnol Bioeng 118:952–962. https://doi.org/10.1002/BIT.27625

Sforza E, Pastore M, Barbera E, Bertucco A (2019) Respirometry as a tool to quantify kinetic parameters of microalgal mixotrophic growth. Bioprocess Biosyst Eng 42:839–851. https://doi.org/10.1007/s00449-019-02087-9

Shoener BD, Schramm SM, Béline F, et al. (2019) Microalgae and cyanobacteria modeling in water resource recovery facilities: A critical review. Water Res X 2:100024. https://doi.org/10.1016/J.WROA.2018.100024

Solimeno A, Samsó R, Uggetti E, et al (2015) New mechanistic model to simulate microalgae growth. Algal Res. 12:350–358

Tang T, Fadaei H, Hu Z (2014) Rapid evaluation of algal and cyanobacterial activities through specific oxygen production rate measurement. Ecol Eng 73:439–445.

https://doi.org/10.1016/j.ecoleng.2014.09.095

Yun YS, Park JM (2003) Kinetic modeling of the light-dependent photosynthetic activity of the green microalga Chlorella vulgaris. Biotechnol Bioeng 83:303–311. https://doi.org/10.1002/bit.10669

THE ANTIOXIDANT POTENTIAL OF MICROALGAL BIOMASS USING THE OXYGEN RADICAL ABSORBANCE CAPACITY (ORAC) METHOD

Valéria Louzada Leal[1,3], Sílvio Augusto Ortolan[2,3], Giséle Alves[1,3], Michele Junkherr Rodrigues[1], Valeriano Antonio Corbellini[1], Rosana de Cassia de Souza Schneider[1,3]

[1]Postgraduate Program in Environmental Technology, University of Santa Cruz do Sul (UNISC), Avenida Independência, 2293, 96815-900, Santa Cruz do Sul, Rio Grande do Sul, Brazil.

[2]Postgraduate Program in Health Promotion, University of Santa Cruz do Sul (UNISC), Avenida Independência, 2293, 96815-900, Santa Cruz do Sul, Rio Grande do Sul, Brazil.

[3] Center of Excellence in Oleochemistry and Biotechnology, Regional Science and Technology Park of the University of Santa Cruz do Sul (Tecno-UNISC) Avenida Independência, 2293 Santa Cruz do Sul, Rio Grande do Sul 96815-900 Brazil.

Correspondence: valeria.louzadaleal@gmail.com

SUMMARY

Antioxidants play a crucial role in protecting cells from damage caused by free radicals and oxidative stress. Microalgae have emerged as a promising natural source of antioxidants due to their ability to produce a wide variety of antioxidant molecules, including polysaccharides, omega-3 and omega-6 fatty acids, carotenoids, and phenolic compounds, within a single cell. The Oxygen Radical Absorption Capacity (ORAC) method has been widely used to evaluate the antioxidant potential of natural raw materials. In this study, the ORAC protocol was presented, using *Euglena gracilis* and *Chlorella sp*. biomass as examples. The results obtained from the ORAC assay provided valuable information on the antioxidant activity of microalgae and demonstrated that this protocol can be applied to other biomasses, serving as a valuable tool for research and development efforts in microalgae-based antioxidant products.

INTRODUCTION

Antioxidants are natural or synthetic compounds that play a crucial role in protecting cells against damage caused by free radicals and oxidative stress, which in excessive levels can lead to cellular and tissue damage and contribute to the development of aging, diabetes, autoimmune, or chronic diseases such as cancer, and cardiovascular and neurological diseases (Coulombier *et al.*, 2021; Koyande *et al.*, 2019). Furthermore, it is important to note that free radicals and oxidative stress can also have detrimental effects on plants, acting in a manner like pathogen infections, leading to the degradation of proteins and lipids, compromising cellular integrity, and adversely impacting plant vitality. This oxidative damage can manifest in physiological symptoms and alterations in plant morphology (Rodrigues de Queiroz *et al.*, 2023).

In response to these damages, there is a growing interest in natural antioxidants due to their non-toxic and non-harmful properties, which make them more desirable than synthetic chemical antioxidants. In this scenario, microalgae are emerging as a promising source of natural antioxidants due to their effectiveness and long-lasting antioxidant properties. These microorganisms have the unique ability to produce a wide range of antioxidant molecules, including polysaccharides, omega-3 and omega-6 fatty acids, carotenoids, and phenolic compounds, all within a single cell. This makes microalgae superior to traditional plant-based sources of antioxidants (Coulombier *et al.,* 2021; Jena & Subudhi, 2019).

In recent years, various methods have been developed to evaluate the antioxidant potential of natural raw materials, including the Trolox Equivalent Antioxidant Capacity (TEAC) assay, Total Radical Absorption Potentials (TRAP) assay, Ferric Reducing/Antioxidant Power (FRAP) assay, and Oxygen Radical Absorption Capacity (ORAC) assay (Ou *et al.,* 2001). Each of these techniques offers a comprehensive understanding of the antioxidative properties of different compounds and is widely employed in antioxidant research and evaluation. Among these methods, the ORAC assay has gained extensive usage for characterizing the antioxidant capacity of both pure and complex compounds. Its popularity has grown to the extent that some manufacturers now incorporate ORAC values in their product descriptions as a measure of antioxidant power (Ou *et al.,* 2001).

The ORAC assay was originally developed by Cao *et al.* (1993; 1995) and later improved by Ou *et al.* (2001). This assay is based on measuring the *in vitro* antioxidant activity. The measurement is conducted at a temperature of 37°C in a buffered medium of potassium phosphate (pH 7.4). Fluorescein is used as a fluorescent probe, while AAPH (2,2-azobis(2-methylpropionamidine) dihydrochloride) serves as the peroxyl radical generator. Additionally, Trolox (6 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble vitamin-E analog, is used as the standard antioxidant. During the ORAC assay, the reaction is monitored by the rate of fluorescent light emitted by the fluorescein in the presence of the antioxidant. As higher the efficacy of the antioxidant compound in eliminating free radicals, than less the decrease in the intensity of fluorescein. On the other hand, the reduction in fluorescence intensity of fluorescein indicates the level of damage caused by its interaction with peroxyl radicals **(Figure 1).** In the presence of an antioxidant, peroxyl radicals (ROO•) react by abstracting a hydrogen atom from the antioxidant, leading to the formation of hydroperoxide (ROOH) and a stable antioxidant radical (ArO•). This reaction mitigates the detrimental effects of peroxyl radicals on fluorescein. The protective ability of an antioxidant is evaluated by comparing the area under the decay curve of fluorescence (AUC) for the sample to that of a blank without any antioxidant (Ou *et al.,* 2001).

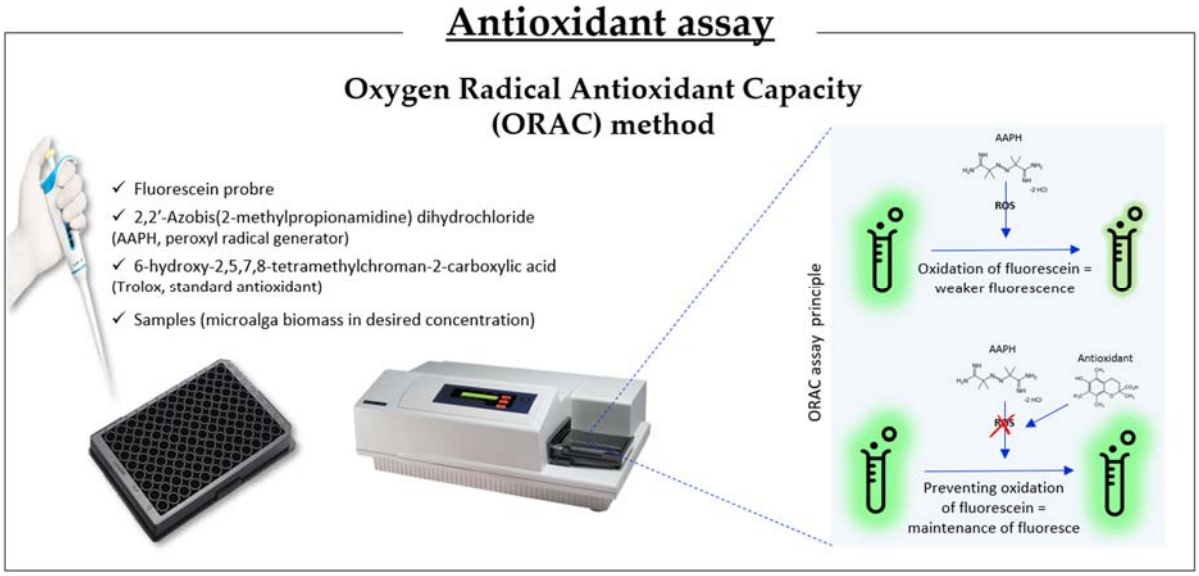


Figure 1. Oxygen Radical Absorption Capacity (ORAC) method principle.

Therefore, we propose using ORAC values as a reference to assess the "peroxyl radical absorption capacity" of microalgae biomass. However, it is important to note that the ORAC assay does not provide a comprehensive evaluation of "total antioxidant activity" as it solely focuses on measuring antioxidant activity against peroxyl radicals. To obtain a comprehensive understanding of the antioxidant activity profile, a comprehensive set of assays is required.

PROTOCOL

Reagents

Potassium phosphate buffer

- Solution 1: Weigh 13.06 g of Potassium Phosphate Dibasic Anhydrous (K_2HPO_4) in a 1000 mL volumetric flask, and complete the volume with ultrapure water.
- Solution 2: Weigh 5.105 g of Potassium phosphate monobasic Anhydrous (KH_2PO_4) in a 500 mL volumetric flask, and complete the volume with ultrapure water.
- Use solution (75 mmol L^{-1}): Transfer solution 1 to a 2000 mL beaker and measure the pH. Gradually add solution 2 (monobasic potassium phosphate) until the solution reaches a pH of 7.4 ± 0.05. This buffer should be prepared simultaneously with the other reagents and used in their preparation.

Fluorescein

- Stock solution (407 μmol L^{-1}): Weigh 6.75 mg (0.00675 g) of fluorescein directly into a 50 mL volumetric flask. Dilute it with 2 mL of absolute ethanol and calibrate the flask using a potassium phosphate buffer. Store the solution under refrigeration and away from light. The validity of the stock solution is ± 6 months.
- Use solution (81 nmol L^{-1}): In a 100 mL volumetric flask, add 50 mL of potassium phosphate buffer. Add 20 μL of the fluorescein stock solution and calibrate the flask again using a potassium phosphate buffer.

Trolox (6 hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid)

- Stock solution (4000 μmol L^{-1}): Weigh 10 mg (0.01 g) of Trolox directly into a 10 mL volumetric flask. Dissolve it (under agitation) with approximately 5 mL of potassium phosphate buffer and calibrate the flask. Use a micropipette to make 1 mL aliquots in properly identified microtubes. Store the microtubes in the freezer. The validity of the stock solution is undetermined.
- Use solution (2 a 96 μmol L^{-1}): Thaw one of the microtubes of the solution stored in the freezer at room temperature. Prepare the following dilutions (Table 1) in 2 mL microtubes, using potassium phosphate buffer as the diluent:

Table 1. Dilutions for Trolox standard analytical curve.

Trolox standard curve				
Trolox Standard_ curve point	**Concentration (μmol L^{-1})**	**Concentration (mg L^{-1})**	**Aliquot from Trolox solution (μL)**	**Potassium phosphate buffer (μL)**
TS_P1	96	25,34	48 from stock solution	1952
TS_P2	64	16,89	400 from point 1	200
TS_P3	48	12,67	250 from pont 1	250
TS_P4	32	8,45	250 from point 2	250
TS_P5	24	6,34	250 from point 3	250
TS_P6	16	4,22	250 from point 4	250
TS_P7	12	3,17	250 from point 5	250
TS_P8	8	2,11	250 from point 6	250
TS_P9	4	1,06	250 from point 8	250
TS_P10	2	0,53	250 from point 9	250

AAPH (2,2-azobis(2-methylpropionamidine) dihydrochloride)

- Use solution (152 mmol L^{-1}): This solution must be prepared at the time of carrying out the test, as close as possible to the preparation of the microplate. Weigh 206 mg (0.206 g) of AAPH directly into a 5 mL volumetric flask. Dissolve with approximately 3 mL of potassium phosphate buffer and calibrate the flask using a potassium phosphate buffer. Validity: up to 4 hours.

Samples

- Stock solution (5,000 mg L^{-1}): Weigh 50 mg of the dry biomass sample and transfer it to a 10 mL volumetric flask. Calibrate the flask with a diluent that solubilizes the sample and is miscible with phosphate buffer (e.g., methanol, ethanol). If necessary, an ultrasound of the flask for 10 minutes.
- Use solution: For the initial test, a test concentration should be used as specified in the literature. Prepare a final volume of 50 mL by diluting the indicated volume of the sample stock solution with potassium phosphate buffer in a volumetric flask.

Assay preparation

The microplate should be a 96-well black polystyrene microplate flat bottom. Additionally, it is recommended to use a multichannel micropipette to speed up the process without compromising the results.

To prepare the microplate, please follow the microplate layout **(Figure 2)** and use the respective volumes of reagents provided below.

Microplate Layout												
	1	2	3	4	5	6	7	8	9	10	11	12
A	PB	PB	FB	FB	RB	RB	BB	BB	BB	TS_P1	TS_P1	TS_P1
B	TS_P2	TS_P2	TS_P2	TS_P3	TS_P3	TS_P3	TS_P4	TS_P4	TS_P4	TS_P5	TS_P5	TS_P5
C	TS_P6	TS_P6	TS_P6	TS_P7	TS_P7	TS_P7	TS_P8	TS_P8	TS_P8	TS_P9	TS_P9	TS_P9
D	TS_P10	TS_P10	TS_P10	S1	S1	S1	S2	S2	S2	S3	S3	S3
E	S4	S4	S4	...	...	...						
F												
G												
H												

Figure 2. The microplate layout for the ORAC assay includes blanks, trolox standard points for the analytical curve, and samples.

Where:

- PB (Plate Blank) = 200 µL of Potassium phosphate buffer (wells A1 and A2);
- FB (Fluorescein Blank) = 50 µL of buffer + 150 µL of fluorescein use solution (wells A3 and A4);
- RB (Radical Blank) = 175 µL of buffer (wells A5 and A6);
- BB (Buffer Blank) = 25 µL of buffer + 150 µL of fluorescein use solution (wells A7, A8 and A9);
- TS (Trolox Standard) = 25 µL of each point of Trolox curve (triplicate) + 150 µL of fluorescein use solution (wells A10 to D3); and,
- S (Sample) = 25 µL of sample + 150 µL of fluorescein use solution (triplicate).

Observation: The PB, FB, and RB are for checking the viability of the plate, and should be done in duplicates; The BB, TS_P1 to TS_P10, and S are the pieces of information that will be used for the calculations and should be done in triplicates.

1) In the microplate reader, incubate the plate for 10 minutes at 37 °C. During the last 3 minutes of incubation, maintain constant agitation;
2) Remove the plate from the equipment and pipette 25 μL of the AAPH use solution into wells A5 to D3, as well as into the sample wells (the final volume in each well should be 200 μL, with final concentrations of 61 nmol L^{-1} fluorescein and 19 nmol L^{-1} AAPH);
3) Reposition the microplate in the equipment, configuring it to the "top read", confirm that the temperature is set to 37 °C, and shake the plate for 30 seconds;
4) Set the microplate reader to monitor fluorescence with an excitation wavelength (λ excitation) of 485 nm and an emission wavelength (λ emission) of 528 nm, every minute, for 91 minutes (1h 31 min) or until it reaches less than 0.5% of the initial fluorescence value (e.g., if the initial fluorescence was 400, it should drop down to 2).

For calculating the final ORAC values, use a linear regression equation (y = ax + b) based on net area under the fluorescence decay curve of the Trolox standard and express the data as micromoles of Trolox equivalents (TE) per gram or per milliliter sample (μmol of TE g^{-1} or μmol of TE mL^{-1}).

RESULTS

In this study, the ORAC (Oxygen Radical Absorbance Capacity) method was applied to evaluate the antioxidant capacity of the biomass of *Euglena gracilis* and *Chlorella* sp. microalgae and illustrate the applications of the method. The results showed that the biomass of *E. gracilis* exhibited an antioxidant capacity of 155.4 ± 18.7 μmol of TE g^{-1}, while the biomass of *Chlorella* sp. achieved an antioxidant activity of 124.2 ± 29.8 μmol of TE g^{-1} (Figure 3).

DISCUSSION

Is well known that microalgae are a rich source of compounds such as carotenoids, phenolic compounds, chlorophyll, and vitamins, all of which possess antioxidant properties (Coulombier *et al.*, 2021; De Souza *et al.*, 2020; Jena & Subudhi, 2019). Consequently, this biomass is increasingly being explored as a natural source of antioxidants applicable to various industries, including food, cosmetics, and pharmaceuticals (Barsanti; Gualtieri, 2019; Chatterjee; Bhattcharjee, 2014).

The Oxygen Radical Absorption Capacity (ORAC) assay, which measures a compound's ability to neutralize free radicals generated *in vitro,* has been employed to evaluate the antioxidant potential of numerous natural raw materials (Ou *et al.*, 2001; Wu *et al.*, 2004), including microalgae (De Souza *et al.*, 2020; Zhao *et al.*, 2020; Banskota et al., 2019).

In this study, the ORAC assay successfully evaluated the antioxidant activity of *Euglena gracilis* and *Chlorella* sp. microalgae biomass. Based on the findings of this study, it can be concluded that the ORAC assay is a dependable method for evaluating the antioxidant potential of microalgae, enabling an accurate and comparative assessment of the antioxidant activity among different microalgae species, aiding in the identification of those with more pronounced antioxidant activity. Such information is critical for the development of industrial applications, including functional foods, cosmetics, and dietary supplements, which can leverage the antioxidant properties of microalgae.

In conclusion, the ORAC assay proves to be a valuable tool for research and development efforts in microalgae-based antioxidant products. It provides essential insights into the capacity to neutralize free radicals, facilitating the selection of promising raw materials within the antioxidant industry.

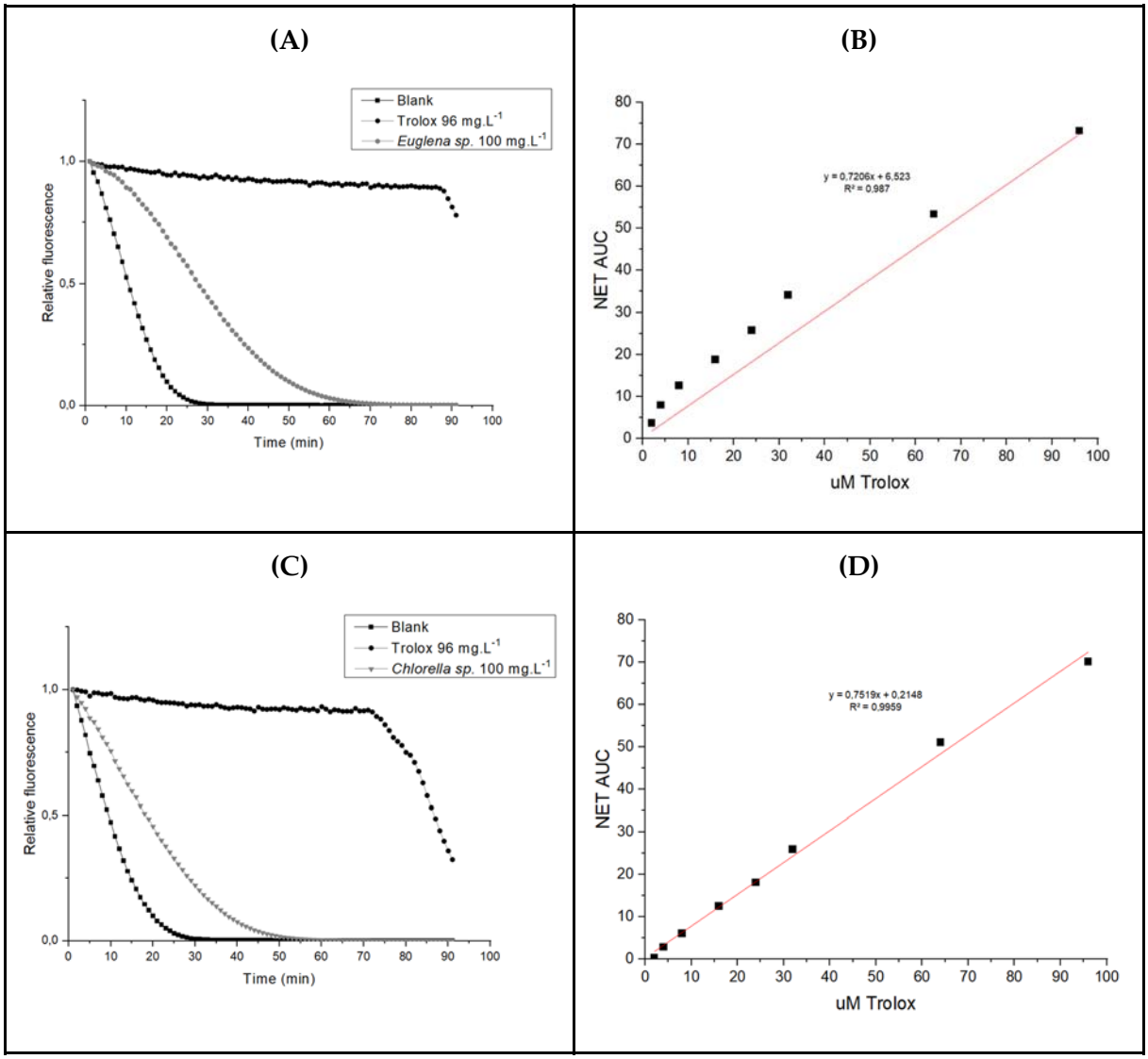


Figure 3. Relative fluorescence **(A)** and analytic curve of *Euglena gracilis* **(B).** Relative fluorescence **(C)** and analytic curve of *Chlorella* sp. **(D).**

ACKNOWLEDGMENTS

Capes code 001, CNPq protocol 306216/2022-1 and protocol 400862/2019-0, SICT-RS process 22/2500-0000201-0.

DISCLOSURES

The authors declare they have no conflicts of interest.

REFERENCES

BARSANTI, Laura; GUALTIERI, Paolo. Paramylon, a potent immunomodulator from WZSL mutant of *Euglena gracilis*. *Molecules*, 2019, vol. 24, no 17, p. 3114.

BANSKOTA, Arjun H., et al. Antioxidant properties and lipid composition of selected microalgae. *Journal of Applied Phycology*, 2019, vol. 31, p. 309-318.

CAO, Guohua; ALESSIO, Helaine M.; CUTLER, Richard G. Oxygen-radical absorbance capacity assay for antioxidants. *Free radical biology and medicine*, 1993, vol. 14, no 3, p. 303-311.

CAO, Guohua, et al. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clinical chemistry*, 1995, vol. 41, no 12, p. 1738-1744.

CHATTERJEE, Dipan; BHATTACHARJEE, Paramita. Supercritical carbon dioxide extraction of antioxidant-rich fraction from *Phormidium valderianum*: Optimization of experimental process parameters. *Algal Research*, 2014, vol. 3, p. 49-54.

COULOMBIER, Noémie; JAUFFRAIS, Thierry; LEBOUVIER, Nicolas. Antioxidant compounds from microalgae: A review. *Marine drugs*, 2021, vol. 19, no 10, p. 549.

DE SOUZA, Maiara P., et al. Bioproducts characterization of residual periphytic biomass produced in an algal turf scrubber (ATS) bioremediation system. *Water Science and Technology*, 2020, vol. 82, no 6, p. 1247-1259.

JENA, Jayanti; SUBUDHI, Enketeswara. Microalgae: An untapped resource for natural antimicrobials. *The role of microalgae in wastewater treatment*, 2019, p. 99-114.

KOYANDE, Apurav Krishna, et al. Microalgae: A potential alternative to health supplementation for humans. *Food Science and Human Wellness*, 2019, vol. 8, no 1, p. 16-24.

RODRIGUES DE QUEIROZ, Aline, et al. The effects of exogenously applied antioxidants on plant growth and resilience. *Phytochemistry Reviews*, 2023, vol. 22, no 2, p. 407-447.

OU, Boxin, et al. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays a comparative study. *Journal of agricultural and food chemistry*, 2002, vol. 50, no 11, p. 3122-3128.

ZHAO, Zhenyu, et al. Identification and screening of multiple tropical microalgal strains for antioxidant activity in vitro. *Food Bioscience*, 2020, vol. 36, p. 100649.

LONG-TERM STORAGE OF PHOTOAUTOTROPHIC MICROORGANISMS ON SOLID CULTURE MEDIUM

Enrique Romero Frasca[1], Germán Buitrón[1]
[1] Laboratory for Research on Advanced Processes for Water Treatment, Unidad Académica Juriquilla, Instituto de Ingeniería, Universidad Nacional Autónoma de México, Blvd. Juriquilla 3001, Querétaro 76230, México
Correspondence: gbuitronm@iingen.unam.mx

SUMMARY

A method for preserving microscopic algae and cyanobacterial cultures in solid media within Petri dishes is presented by taking advantage of the chlorophyll retention mechanism without the illumination of the microorganisms.

INTRODUCTION

Bioprospecting photoautotrophic microorganisms such as microalgae and cyanobacteria have emerged as an important line of study in engineering and environmental sciences. Currently, there is a vast catalog of species with significant and valuable biotechnological applications [1], ranging from pigment production (*i.e.*, chlorophyll or carotenoids) to the generation of liquid or gaseous biofuels and the manufacturing of biofertilizers, bioplastics, etc. As a result, proper long-term conservation of these species in the laboratory is vital for developing any research project, as it ensures the preservation of its genetic traits and avoids cross-contamination of the surrounding environment. While cryopreservation has been established as the traditional method to preserve algal species for over five years, viability assays have demonstrated that this method can result in irreversible damage to the cell membrane [2]. Consequently, the search for novel strategies to preserve microalgae or cyanobacteria that could be easy to operate, less cell abrasive, and cost-effective has increased. Fundamentally, microalgae and cyanobacteria can regulate their chlorophyll production through protochlorophyllide oxidoreductase [3], an enzyme responsible for converting protochlorophyllide into chlorophyllide, a primary precursor of chlorophyll. Considering that the activity of protochlorophyllide oxidoreductase is light-dependent, it has been observed that chlorophyll production and cell aging (senescence) are notably decreased under prolonged darkness [4]. A methodology for preserving previously isolated microalgae and cyanobacteria is presented using such latency during cellular development under dark conditions.

MATERIALS AND REAGENTS

Name	Vendor	Cat #	Comments
Sulfuric acid, A.C.S, ≥95.0%	Herschi Trading	A1590	
Agar-Agar	Thermo Scientific™	9002-18-0	
Inoculation loop			
Explorer® analytical balance	Ohaus	21147	d=0.1 mg
Plastic tray			Dimensions: 57 x 14 mm
Standard Spoon Spatula	Bochem™	6078270	Stainless steel 18/10
Volumetric flask, Class A	VelaQuin	1621A-250	Volume size: 250 mL
		1621A-500	Volume size: 500 mL
Bunsen burner			
Research Plus® Adjustable Volume, Single Channel Pipette	Eppendorf	3123000055	Volume size: 20 - 200 µL
Petri dishes			
Measuring cylinder, Class A	VelaQuin	1601P-250	Volume size: 250 mL
Pipette tips	Corning	CLS4860	1-200 µL universal fit
Cotton plugs			

PROCEDURE

First, wash the glassware to be used with neutral soap. Rinse thoroughly with water to remove excess soap and, if possible, finish rinsing with 2-4%v/V H_2SO_4. Allow the glassware to dry entirely upside down -or in a way that prevents contact with the air- before proceeding with the method.

1. Prepare the recommended solid culture medium for the isolated cyanobacteria or microalgae strain. The following growth media are recommended for cyanobacteria (BG-11), freshwater microalgae (Basal Bold Medium or BBM), *Spirulina* (Zarrouk), diatoms and coastal seaweeds (f/2), and marine algae (Artificial Seawater or ASN-III). Sterilize at 15 psi (121°C) for 30 minutes if needed.

2. Disinfect a designated workspace using one of the following solutions: i) 70% isopropyl alcohol (IPA), ii) 80% ethanol, or iii) 5% sodium hypochlorite (NaOCl). Then, place the strain to be inoculated and sterilized glassware (Petri dishes, sterilized medium, inoculation loop, etc.) within a 15-25 cm radius from the Bunsen burner flame and wait about 10 minutes before starting. Always work behind the flame, avoid talking or exhaling abruptly during handling or inoculation, and use a face mask to ensure aseptic conditions.

3. Pour enough culture medium into a Petri dish to reach one-third of the total container height. Allow it to solidify at room temperature within the sterilized area and on a flat surface.

4. Inoculate the desired strain as follows:

    4.1. - If the strain is on a solid medium, allow the inoculation loop to heat up until red-hot in the Bunsen burner flame and let it cool behind the flame. Take a sample using the loop's tip and streak the loop in a zig-zag pattern on the culture medium surface. Seal the Petri dish with parafilm and masking tape when finished.

    4.2. - If the strain is in a liquid medium, retrieve a known volume of the strain using a previously cleaned pipette behind the Bunsen burner. Empty the sample into the Petri dish, close the lid, and gently spread the liquid in circular movements by gently tilting the Petri dish around the work table. Seal with plastic wrap and masking tape.

5. Finally, incubate the Petri dishes at room temperature in a dark room. When inoculum is needed for further experiments, transfer dark-incubated Petri dishes to an environment with low photosynthetically active radiation (1 to 4 $\mu mol\ m^{-2}\ s^{-1}$) for 72 hours and then to 13-55 $\mu mol\ m^{-2}\ s^{-1}$ until growth is observed.

RESULTS

The eukaryotic green algae *Chlorella vulgaris* was streaked on a solid BBM medium (Fig. 1a) and preserved in complete darkness for approximately three months. After 14 days of being transferred to an environment with moderate light (Figure 1c), the strain covered almost the entire Petri dish, as observed by their characteristic green color (chlorophyll). It is worth noting that no morphological differences were observed in *C. vulgaris* under the optical microscope before (Figure 1b) and after (Figure 1d) its preservation in darkness.

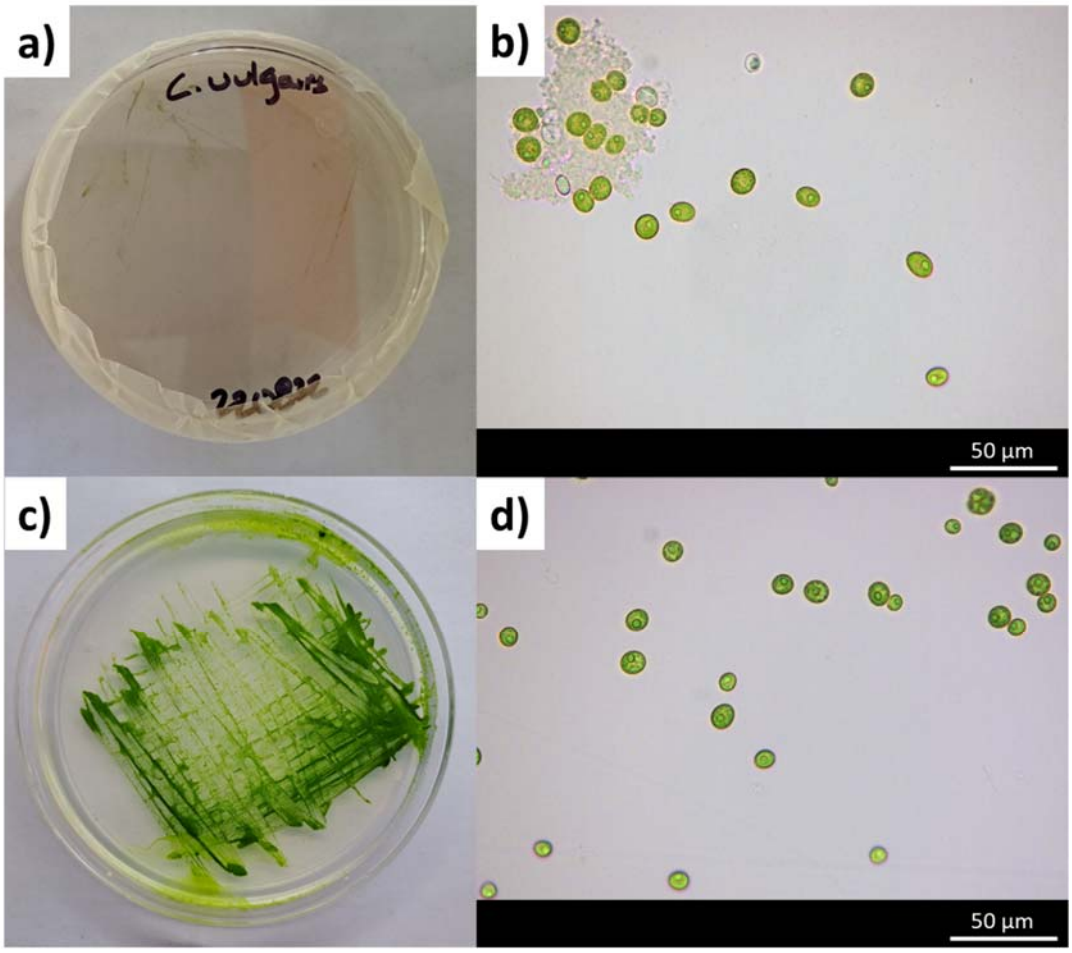


Figure 1. *Chlorella vulgaris* on BBM before dark incubation (a) and 14 days after photosynthetic reactivation under moderate light (c). Morphological characteristics under the optical microscope at 30X before incubation in complete darkness (b) and after photosynthetic reactivation (d).

DISCUSSION

It is well known that microalgae and cyanobacteria enter a state of cellular paralysis or senescence during prolonged periods of darkness, where chlorophyll is degraded as a survival mechanism (chlorosis). However, it has been observed that specific genera like *Chlorella*, *Scenedesmus*, *Coelastrum*, and *Monoraphidium* still retain an ancient light-independent metabolic pathway for chlorophyll biosynthesis, catalyzed by the light-independent enzyme protoporphyrinogen oxidoreductase (DPOR) [5]. Such enzyme, older than its light-dependent counterpart, was a metabolic response of early photosynthetic microorganisms, enabling the cell not only to continue chlorophyll production during extended periods of darkness and low oxygen concentration but also to assist in the reactivation of the cellular light-harvesting complex and thus cellular growth, in the case of a gradual increase in photosynthetically active radiation [6].

ACKNOWLEDGMENTS

The support from CYTED through the RENUWAL Network Project P319RT002 is appreciated. E. Romero-Frasca acknowledges the funding provided by the CONACYT-OAS-AMEXCID Scholarship Program (No. 855766).

REFERENCES

[1] Mutanda T, Naidoo D, Bwapwa JK, Anandraj A (2020). Biotechnological Applications of Microalgal Oleaginous Compounds: Current Trends on Microalgal Bioprocessing of Products. Frontiers in Energy Research 8, 1-21. https://doi.org/10.3389/fenrg.2020.598803.

[2] Bui TVL, Ross IL, Jakob G, Hankamer B (2013). Impact of Procedural Steps and Cryopreservation Agents in the Cryopreservation of Chlorophyte Microalgae. PLoS One 8, e78668. https://doi.org/10.1371/journal.pone.0078668.

[3] Carmo Da Silva J, Lombardi AT, Da Silva JC , Lombardi AT (2020). Chlorophylls in Microalgae: Occurrence, Distribution, and Biosynthesis. In: Jacob-Lopes E, Queiroz MI, Zepka LQ (Ed) Pigments from Microalgae Handbook. Springer Nature, Cham. https://doi.org/10.1007/978-3-030-50971-2_1.

[4] Vedalankar P, Tripathy BC (2018). Evolution of light-independent protochlorophyllide oxidoreductase. Protoplasma 256, 293–312, https://doi.org/10.1007/S00709-018-1317-Y.

[5] Heyes DJ et al. (2021). Photocatalysis as the 'master switch' of photomorphogenesis in early plant development. Nature Plants 7, 268–276. https://doi.org/10.1038/s41477-021-00866-5.

[6] Yee W., Tang SGH, Phua PSP, Megawarnan H (2019). Long-term maintenance of 23 strains of freshwater microalgae on solid microbiological culture media: A preliminary study. Algal Research 41, 101516. https://doi.org/10.1016/j.algal.2019.101516.

A SIMPLE AND ALTERNATIVE SCREENING METHOD TO RAPIDLY ASSESS ENZYMATIC HYDROLYSIS OF ALGAL BIOMASS

Rafael Vitorino[1,3], Bernardo Carvalho[1], Daniel Figueiredo[1], Alice Ferreira[2], Hugo Pereira[1], João Varela[1,3], Luisa Gouveia[1,2]

[1] GreenCoLab - Green Ocean Technologies and Products Collaborative Laboratory, CCMAR, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal

[2] LNEG_UBB - Laboratório Nacional de Energia e Geologia IP. - Unidade de Bioenergia e Biorrefinarias, Estrada do Paço do Lumiar, 22, 1649-038, Lisboa, Portugal

[3] CCMAR - Centre of Marine Science, University of Algarve, Campus de Gambelas, 8005-139, Faro, Portugal

Correspondence: luisa.gouveia@lneg.pt

SUMMARY

Enzymatic hydrolysis is a multi-purpose method used in various industrial fields for many potential applications. However, efficiently employing this methodology can be challenging due to differences in the biochemical composition of the biomass, or the specifications of the desired targeted compounds. Usually, lab-scaled stirred tank bioreactors are utilized to evaluate the hydrolytic activity of enzymes. Still, these protocols are often complex and time-consuming, making it difficult to properly assess the potential of these enzymes against the starting product. In this chapter, we propose a simple and quick alternative method to evaluate the hydrolytic activity of several enzymes in algal biomass. The method is based on incubating enzymes and small volumes of biomass in microplates using orbital shaking and controlled temperature. This protocol can evaluate the hydrolytic activity of different types of enzymes simultaneously in less than 24 hours. In conclusion, this method is a practical approach to quickly test different hydrolysis conditions, decreasing the costs of evaluation assays for downstream applications.

INTRODUCTION

Enzymatic hydrolysis is a biochemical reaction responsible for the breakdown of a target compound (also known as substrate), following its interaction with a hydrolytic enzyme [1]. It is often utilized in various industrial and research fields due to its broad-range applicability, including cellular disruption and compound extraction, production, degradation, or modification [2–7]. For example, a study based on oil extraction following cellular disruption on *Nannochloropsis gaditana* discovered

that coupling enzymatic hydrolysis with an ultrasound pre-treatment enhanced oil extraction [8]. In the biofuel industry, enzymatic hydrolysis is also used to convert cellulosic and lignocellulosic biomass into glucose, to improve the subsequent fermentation step that converts glucose into bioethanol [9]. Hydrolytic enzymes are also commonly used for the chemical modification of target compounds to alter their properties. For example, one study revealed that enzymes that modify post-synthesis polyesters have the potential to enhance the physical and thermal properties of these materials while maintaining their biodegradability [10]. Another practical application of enzymatic hydrolysis is its usage for more sustainable disposal of biopolymers [6].

To ensure the performance of enzymes, it is important to consider the efficiency of the biochemical reactions, which greatly depends on the interaction between the hydrolytic enzyme and the target compound. These reactions can be influenced by both internal and external factors, such as temperature, pH, viscosity, inhibitors, and activators, or molecular affinity, size, concentrations, and cofactors, respectively [11]. As such, a thorough analysis of the working conditions of each enzyme and their interaction with the biomass is necessary to achieve the desired hydrolysis rate [11,12]. Reliable methods to assess enzymatic hydrolysis performance are key to achieving efficient enzymatic bioprocesses. However, most are expensive and time-consuming, impacting research and successful process optimization [12]. Through the proposed method in this chapter, we can evaluate the enzymatic hydrolysis via a fast-pre-screening approach using 12 or 24 well microplates, providing a potential solution to alleviate the challenges by reducing the necessary time and laboratory resources and increasing the throughput of enzymatic performance assessment. The subsequent degree of hydrolysis can then be measured through a multitude of analytical methods, including compound release (proteins, amino acids, lipids, reducing sugars, etc.) and turbidity variations (absorbance at 750 nm), depending on the desired final application [13–15].

PROTOCOL

Materials

- 12 or 24 well microplates;
- Buffer solutions for pH adjustment;
- Micropipettes;
- 15mL falcons, 2mL or 5mL Eppendorf;
- Resuspended biomass at a known concentration, twice the concentration expected for the assay;
- Orbital incubators with temperature control.

Experimental Procedure

1. Plan the microplate layout considering a random sample order to minimize bias (see the example in Figure 1). Wells at the edge of the microplate should be filled with water to minimize evaporation in the treatment wells;
2. Pipette the biomass suspension of each sample into their respective wells (at double the concentration you wish to test). Use 1.75 mL for a 12-well plate and 1.0 mL for a 24-well plate;
3. Pipette each buffer into the respective wells. Use 1.75 mL for a 12-well plate and 1.0 mL for a 24-well plate;
4. Add the enzymes to the respective treatment wells;
5. Fill the empty wells with water. Use 3.5 mL for a 12-well plate and 2.0 mL for a 24-well plate;
6. Close the microplates and seal them sideways with parafilm;
7. Place the microplates in orbital incubators previously set to the desired hydrolysis temperature;
8. Incubate microplates for no longer than 24 hours to prevent excessive evaporation in the treatment wells;
9. Once the incubation period is over, you can directly analyze the enzymatic hydrolysis of the recovered samples through turbidity measurements (absorbance at 750nm) or other relevant analytical methods (e.g., the compound release of proteins, amino acids, lipids, etc.);
    a. (Optional) Alternatively, you can recover the samples of each well to 15mL falcons or 5mL/2mL microcentrifuge tubes (Note: Make sure to thoroughly resuspend each well before transferring the sample) and store the samples for future analysis.

REPRESENTATIVE RESULTS

In the provided results, we utilized *Raphidonema monicae* biomass as the target for hydrolysis, and we selected alcalase (Merck Millipore), cellulase (Sigma-Aldrich), mannanase (Sigma-Aldrich), viscozyme (Sigma-Aldrich) and pectinase (Novozymes) as the hydrolytic enzymes, at 8% w/w. For pH 8 enzymatic reactions, potassium phosphate buffers at 0.1 mmol were used, while pH 4 and 6 enzymatic reactions were buffered with citrate buffers also at 0.1 mmol. The procedure was performed twice, simultaneously, once in 12-well plates and another in 24-well plates, in biological duplicates, overnight, at 50 ^{0}C, to assess the replicability of the method across different microplates. A control was included in both procedures, which excluded the addition of enzymes, to evaluate if the temperature itself (50 ^{0}C) had any influence on the hydrolysis of the biomass. The analysis was carried out by measuring the decrease in

turbidity from the original non-treated biomass to the treated samples, by reading the optical density (OD) at 750nm. As the biomass is hydrolyzed and the particles in suspension decrease in size, we expect to observe a decrease in the measured absorbance, with more extensive hydrolysis resulting in a lower OD at 750nm.

We observed that, by exposing the biomass to different hydrolytic enzymes (alcalase, cellulase, mannanase, viscozyme, and pectinase), there was a variation of turbidity between samples (Figures 3 and 4). Depending on the enzyme utilized, higher or lower variations were observed, with a starting OD of 2.99 decreasing as much as 55.9/58.7% (12/24-well plate) and as little as 8.5/8.2% (12/24-well plate).

In the 12-well plate (Figure 3), the sample subjected to alcalase had the highest decrease in turbidity, 55.9% (from 2.99 to approximately 1.32 OD at 750nm). Meanwhile, the sample treated with mannanase decreased the least in OD (8.5%, decreasing from 2.99 to 2.74). The remaining 3 enzymes (cellulase, viscozyme, and pectinase) all had a similar hydrolytic efficiency, with an OD decrease between 15.5 to 27.7%. The temperature at 50 ˚C seemingly also played a role in cellular disruption, decreasing the OD by approximately 13.2%. It was also noted that the initial and final sample volume was also maintained in the treatment wells (Figure 5).

In the 24-well plate (Figure 4), similar results were obtained to the ones in the 12-well plate. The enzymatic treatment with alcalase had the highest decrease in turbidity. The starting OD was noted to have decreased as much as 58.7%, from an OD of 2.99 to 1.24. Likewise, mannanase had the lowest hydrolytic activity in the targeted biomass, and subsequently the lowest decrease in OD (8.2%, decreasing from 2.99 to 2.75). In the temperature control sample, OD was decreased by 15.1%. No loss of volume was obtained in the treatment wells for both the 24-well and 12-well plates (Figure 6).

The equivalent results shared by both types of plates suggest that this method is potentially replicable across different plates and scaled up to larger volumes of samples while displaying minimal variability between wells of similar enzymatic treatments. In addition, significant variability was also detected among wells of different enzymatic treatments. Overall, these results suggest that this method successfully provided the necessary conditions for the enzymes to carry out their hydrolytic activities to evaluate potential interactions of interest with the selected biomass.

FIGURE AND TABLE LEGENDS:

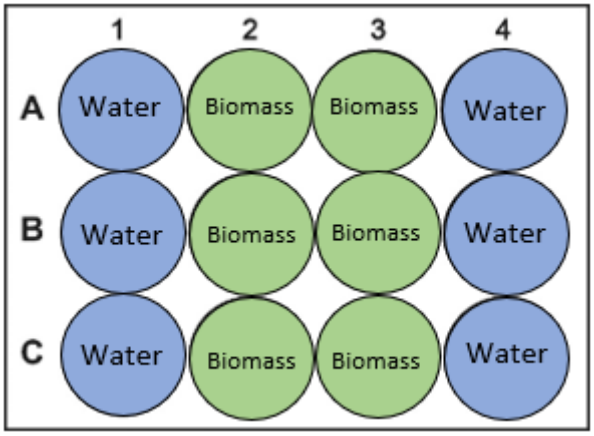


Figure 1. Schematic representation of the biomass and water distribution in a 12-well plate.

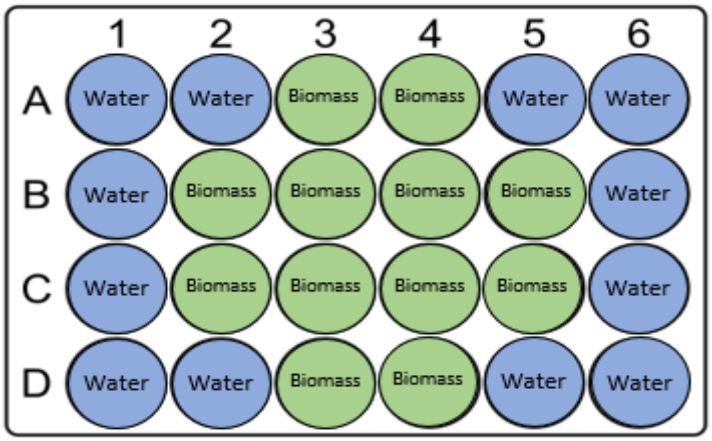


Figure 2. Schematic representation of the biomass and water distribution in a 24-well plate.

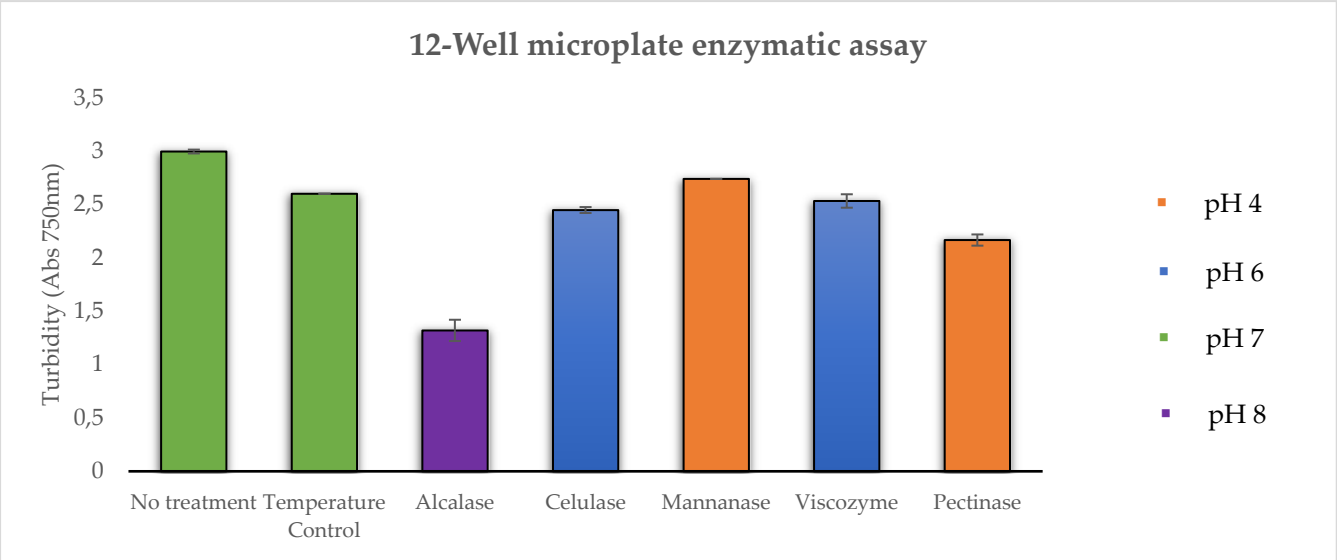


Figure 3. Turbidity (Absorbance at 750nm) results after the enzymatic hydrolysis assay in a 12-well microplate. The error bars represent the maximum and minimum values obtained from biological replicates (*n*=2).

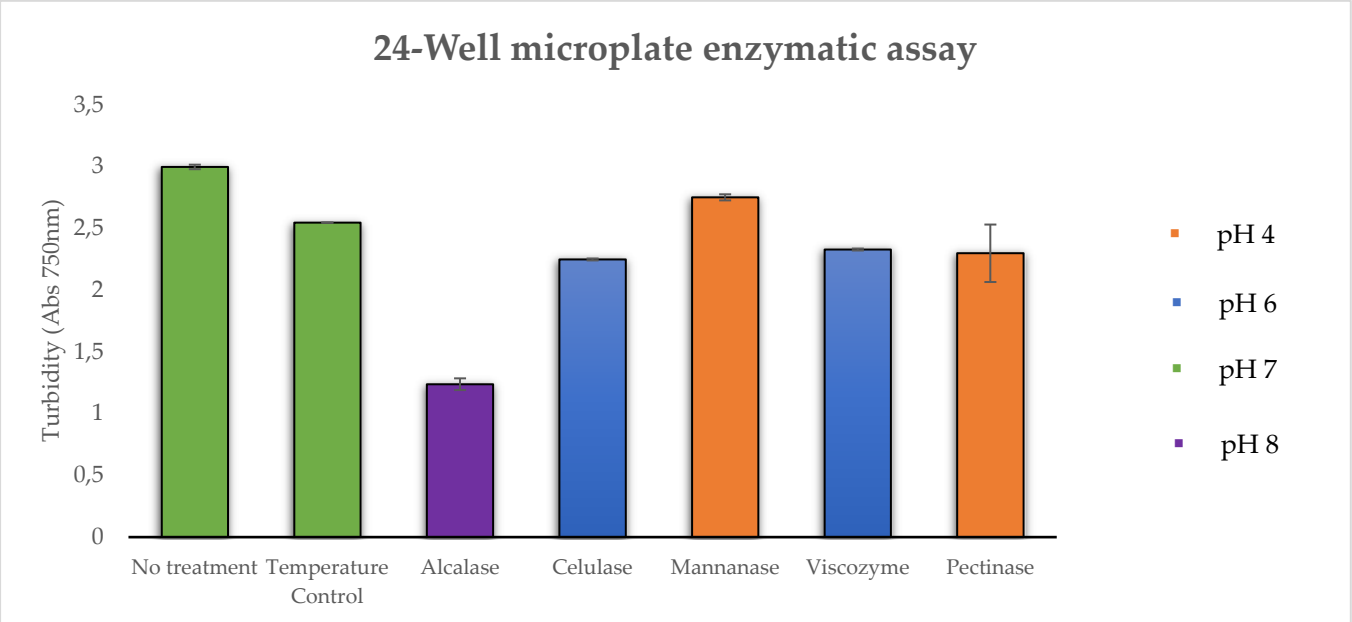


Figure 4. Turbidity (Absorbance at 750nm) results after the enzymatic hydrolysis assay in a 24-Well microplate. The error bars represent the maximum and minimum value obtained from biological replicates (*n*=2).

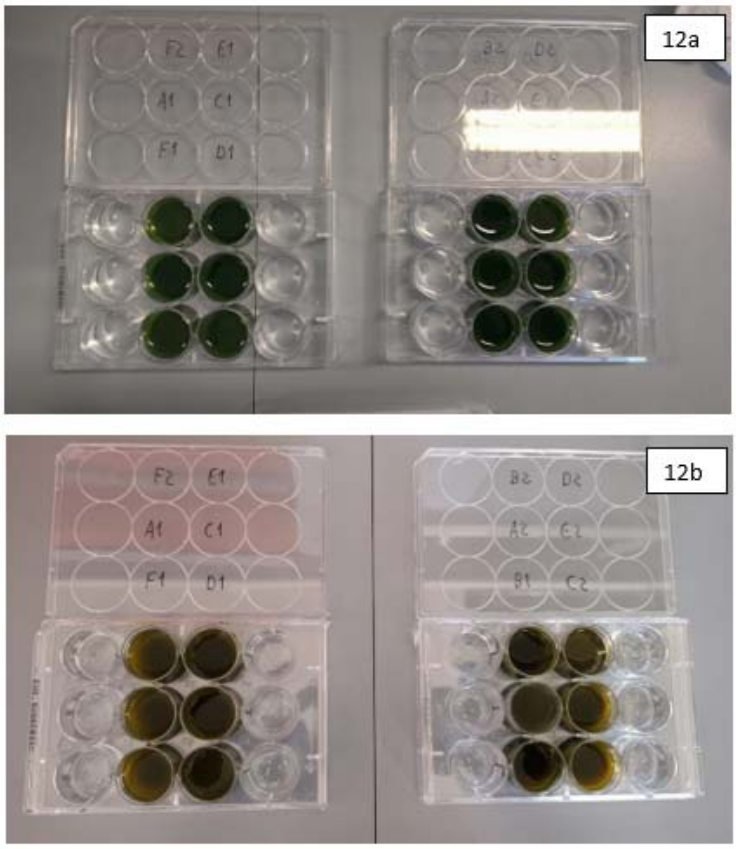


Figure 5. 12-well microplate before (12a) and after (12b) the enzymatic hydrolysis assay.

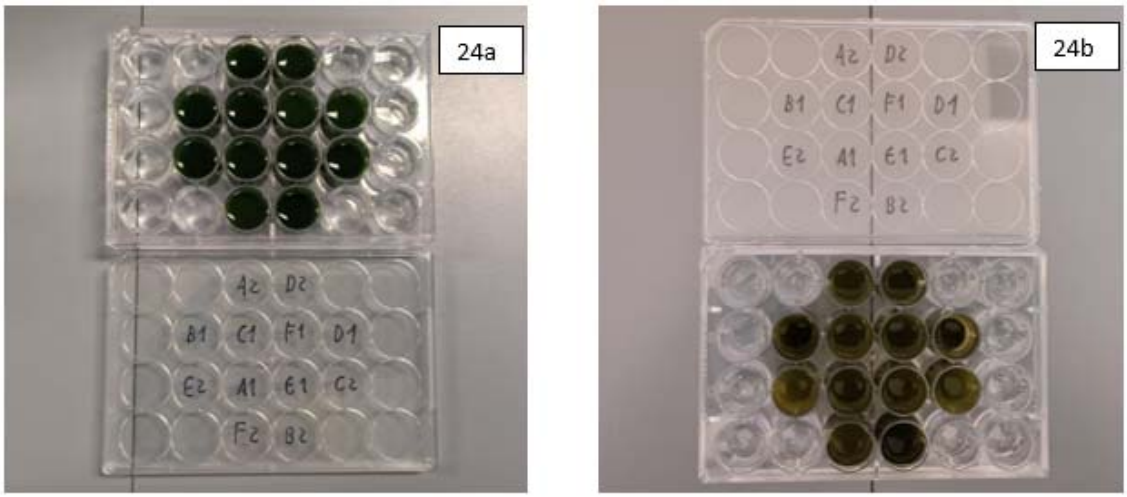


Figure 6. 24-well microplate before (24a) and after (24b) the enzymatic hydrolysis assay.

DISCUSSION

Evaluating the hydrolytic activity of enzymes in lab-scaled stirred tank bioreactors enables the user to test a higher number of conditions, for example, testing enzymes separately, in combination, or sequentially, and in a controlled environment. However, in this situation, these methods are limited both in their complexity, the number of enzymes one can evaluate during each assay, its duration, and the resources used to run the protocol [12]. While these lab-scaled reactors provide more robust and accurate results, the alternative protocol provided in this chapter offers a fast-pre-screening analysis of the hydrolytic activity of various enzymes, economizing both the time and resources spent to run the assay. Nevertheless, this approach also has bottlenecks, including the limited sample volume one can recover from the assay (3.5 mL from a 12-well plate or 2 mL from a-24 well plate), which in turn limits the number of analytical evaluations as the available sample volume is lower. Additionally, the inability to precisely control the reaction conditions in this method suggests that the lab-scaled stirred tank bioreactor method is a better alternative if the user aims to evaluate the enzymatic hydrolysis thoroughly. Nonetheless, the fact that this microplate-based pre-screening method offers the potential to test several samples within a short time frame makes it a worthy alternative if the aim is to evaluate the hydrolytic activity of the selected enzymes in a limited analytic context. However, to ensure bias and sample variability are kept to a minimum, and the resolution is kept within acceptable parameters (parameters to be defined by the user), we recommend special attention and care to the randomization of sample distribution between wells.

In conclusion, this protocol is recommended for users who require a fast enzymatic hydrolysis assay. Lower accuracy and robustness should be expected as this method is not advisable for detailed, in-depth evaluations of the hydrolytic activity of enzymes. Following this assay, once the ideal enzymes have been identified, it is advised for the user to run further validation assays in other more robust lab-scaled bioreactors, such as the stirred tank bioreactors, to evaluate the extent of hydrolysis of the targeted biomass properly.

ACKNOWLEDGMENTS:

This work was financially supported by: (i) PERFORMALGAE – ALG-01-0247-FEEDER-069961 supported by national funds (COMPETE2020, CRESC, Algarve 2020) through ERDF; (ii) Red CYTED P319RT0025 – RENUWAL – Red Iberoamericana para el tratamiento de Efluentes con Microalgas. Alice Ferreira, Daniel Figueiredo, and Bernardo Carvalho are pleased to acknowledge their PhD grants

SFRH/BD/144122/2019, FCT 2022.11872.BDANA and 2022.12615.BDANA, respectively, awarded by Fundação para a Ciência e Tecnologia.

DISCLOSURES:

The authors declare they have no conflict of interest.

REFERENCES

[1] Asif, M.B., Hai, F.I., Jegatheesan, V., Price, W.E., Nghiem, L.D. and Yamamoto, K. (2019) Applications of Membrane Bioreactors in Biotechnology Processes. *Current Trends and Future Developments on (Bio-) Membranes: Membrane Processes in the Pharmaceutical and Biotechnological Field*, Elsevier. 223–57. https://doi.org/10.1016/B978-0-12-813606-5.00008-7

[2] Robinson, P.K. (2015) Enzymes: principles and biotechnological applications. *Essays in Biochemistry*, Portland Press Ltd. **59**, 1–41. https://doi.org/10.1042/BSE0590001

[3] Krakowska-Sieprawska, A., Rafinska, K., Walczak-Skierska, J. and Buszewski, B. (2020) The influence of plant material enzymatic hydrolysis and extraction conditions on the polyphenolic profiles and antioxidant activity of extracts: A green and efficient approach. *Molecules*, MDPI AG. **25**. https://doi.org/10.3390/molecules25092074

[4] Demuez, M., Mahdy, A., Tom As-Pej, E., Gonz Alez-Fern Andez, C. and Ballesteros, M. (2015) Enzymatic Cell Disruption of Microalgae Biomass in Biorefinery Processes. *Biotechnol Bioeng*, **112**, 1955–66. https://doi.org/10.1002/bit.25644/abstract

[5] Glusac, J. and Fishman, A. (2021) Enzymatic and chemical modification of zein for food application. *Trends in Food Science & Technology*, Elsevier. **112**, 507–17. https://doi.org/10.1016/J.TIFS.2021.04.024

[6] Niaounakis, M. (2013) Disposal. *Biopolymers Reuse, Recycling, and Disposal*, William Andrew Publishing. 107–50. https://doi.org/10.1016/B978-1-4557-3145-9.00004-X

[7] Yuan, Y., Jiang, B., Chen, H., Wu, W., Wu, S., Jin, Y. et al. (2021) Recent advances in understanding the effects of lignin structural characteristics on enzymatic hydrolysis. Biotechnol Biofuels. BioMed Central Ltd. https://doi.org/10.1186/s13068-021-02054-1

[8] Blanco-Llamero, C., García-García, P. and Señoráns, F.J. (2021) Combination of synergic enzymes and ultrasounds as an effective pretreatment process to break the microalgal cell wall and enhance algal oil extraction. *Foods*, MDPI AG. **10**. https://doi.org/10.3390/foods10081928

[9] Vasić, K., Knez, Ž. and Leitgeb, M. (2021) Bioethanol production by enzymatic hydrolysis from different lignocellulosic sources. Molecules. MDPI AG. https://doi.org/10.3390/molecules26030753

[10] El-Malek, F.A. and Steinbüchel, A. (2022) Post-Synthetic Enzymatic and Chemical Modifications for Novel Sustainable Polyesters. Front Bioeng Biotechnol. Frontiers Media S.A. https://doi.org/10.3389/fbioe.2021.817023

[11] McDonald, A.G. and Tipton, K.F. (2022) Parameter Reliability and Understanding Enzyme Function. Molecules. MDPI. https://doi.org/10.3390/molecules27010263

[12] Bisswanger, H. (2014) Enzyme assays. *Perspectives in Science*, Elsevier BV. **1**, 41–55. https://doi.org/10.1016/j.pisc.2014.02.005

[13] Benítez Benítez, R., Elvira Tabares, W.F., Lenis Velásquez, L.A., Hurtado Sánchez, C.I. and Salinas Cruel, O.A. (2021) Enzymatic hydrolysis as a tool to improve total digestibility and techno-functional properties of pigeon pea (Cajanus cajan) starch. *Heliyon*, Elsevier Ltd. **7**. https://doi.org/10.1016/j.heliyon.2021.e07817

[14] Rutherfurd, S.M. Methodology for Determining Degree of Hydrolysis of Proteins in Hydrolysates: A Review [Internet].

[15] O'Loughlin, I.B., Murray, B.A., Kelly, P.M., Fitzgerald, R.J. and Brodkorb, A. (2012) Enzymatic hydrolysis of heat-induced aggregates of whey protein isolate. *Journal of Agricultural and Food Chemistry*, American Chemical Society. **60**, 4895–904. https://doi.org/10.1021/JF205213N/ASSET/IMAGES/MEDIUM/JF-2011-05213N_0006.GIF

ELECTROFUSION IN CHLORELLA VULGARIS (CHLORELLACEAE) MICROALGUE STRAINS ISOLATED IN COSTA RICA.

Francinie Murillo-Vega[1], Wayner Montero-Carmona[2], Karla Meneses-Montero[1] & Maritza Guerrero-Barrantes[3]

[1]Technological Institute of Costa Rica, Biotechnology Research Center, Biology Department. Cartago; Costa Rica; frmurillo@itcr.ac.cr, kmeneses@tcr.ac.cr, fvillalta@itcr.ac.cr

[2]Technological Institute of Costa Rica, Agronomy Department, Alajuela, Costa Rica; wmontero@itcr.ac.cr

[3]Algalife Corp, Research Department, San José, Costa Rica; mguerrero@algalife.bio

ABSTRACT

Microalgae such as *Chlorella vulgaris* have been cataloged as highly promising species at the biotechnological level, due to the potential of the genus to express high level recombinant proteins with all the post-translational modifications, grow at low cost (under autotrophic, mixotrophic and heterotrophic conditions) and provide products considered of industrial interest. In order to establish an efficient protocol for microalgae fusion; the conditions of *C. vulgaris* cell wall digestions were established. The alignment conditions were optimized (U1–U2) by variations on the AC voltages used. Finally, the fusion conditions were established (Un) when defining the DC voltages, as well as the amount of pulses, for electrofusion. Through the application of cellulase and macerozyme, electrofusion was facilitated by digesting the cell wall. The application of the established electrofusion parameters and the microselection of the fusion events achieved growth rates of 0.8 gL^{-1} at nine days of growth in a BG-11 culture media.

Keywords: Cell wall digestion, Electrofusion, Microalgae, Microselection.

INTRODUTION

The microalgae *Chlorella vulgaris* Beyerinck (1890) and other *Chlorella* species have been classified as highly promising species for transformation due to the genus's potential to express high molecular weight recombinant proteins with all post-translational modifications. They can grow at low cost (under autotrophic, mixotrophic, and heterotrophic conditions) and provide products considered safe and more market-accepted compared to other biofactories like bacteria (Gong et al. 2011,

Almeida et al. 2017). Additionally, *C. vulgaris* strains have been identified as phytoremediation microalgae for capturing heavy metals and highly concentrated nitrogenous species. Therefore, they also find applications in wastewater treatment and metal removal (Wang et al. 2010, Kumar et al. 2015, Yang et al. 2015).

The rapid growth rate, utilization of non-agricultural land, and efficiency in the production of biotechnological products and processes have positioned this microalga genus as a candidate for renewable bio input production (Fan et al. 2015), being considered a living factory (Specht et al. 2010). Alongside the natural biological potential of the microalga, biotechnological and molecular studies have enhanced the production of proteins, lipids, and other commercially valuable substances in *C. vulgaris* through genetic transformation and metabolic pathway modification (Talebi et al. 2013).

Protoplast induction (cell without cell wall) and its fusion have been studied as genetic improvement techniques, developed in *Chlorella* sp. since 1975 by adapting some protocols previously tested in plants. Protoplast fusion involves the somatic hybridization of two or more cells lacking a cell wall, assisted by chemical or physical methods, generating cellular variants with extra copies of genetic material after the cell regeneration process. This has proven effective in increasing nutrition and the production of valuable metabolites. Preliminary research has studied the structure of the *Chlorella* genus cell wall, demonstrating heterogeneity in morphology and chemical composition even among strains. Scanning electron microscopy revealed different structures in the *Chlorella* sp. cell wall, classified into types I, II, and III based on the number of layers (one or two) and the type of structure (microfibrillar or tri laminar). The importance of this structure in the protoplast transformation process has been highlighted (Yamada and Sakaguchi, 1982; Gerken et al. 2013).

Enzymes were preferred for degrading the cell wall of *C. vulgaris*. Braun (1975) applied pretreatments with cellulases and hemicellulases, achieving up to 80% growth in actively damaged cells with morphological evidence of cell wall damage or complete degradation after a 90-hour incubation period. Subsequent studies evaluated the protoplast regeneration viability of *C. ellipsoidea* and *C. saccharophila*, with survival percentages of 32%, and evidence of subsequent chemically induced cell fusion with polyethylene glycol and Ca^{2+} (Yamada and Sakaguchi 1981, Göbel and Aach, 1985).

Physical methods for protoplast fusion were later studied, with electrofusion described as the application of an electric pulse to align and fuse phospholipid membranes, achieving high efficiency in the process and obtaining larger and more stable cells (Rems et al. 2013). According to Rivera et al. (2014), wavelength, energy, and the duration of the electric field are variables to consider for the success of the

procedure. This process is divided into three successive phases: alignment (U1), fusion (Un), and post-alignment (U2), with each phase utilizing alternating and direct current to facilitate cell migration, membrane porosity, and cytoplasmic union. Electrofusion offers advantages over chemical fusion processes, being a simple, fast, and cost-effective procedure. However, specific procedures need to be developed according to cell type to achieve favorable results in obtaining fused cells and their subsequent growth under laboratory conditions (Rivera et al. 2014). In this study, we assessed conditions for cell wall degradation, alignment (U1-U2), fusion (Un), microselection, and cultivation of resulting fusion events to optimize parameters for electrofusion between cells of *Chlorella vulgaris*.

PROCEDURE

The protocol for electrofusion of *Chlorella vulgaris* microalgal cultures was developed at the Plant Biotechnology Laboratory of the Agronomy Department, Costa Rica Institute of Technology, San Carlos Campus. An axenic strain of *Chlorella vulgaris* was used, identified molecularly with 100% alignment to GenBank accession number X16579.1. The strain is stored in the Microalgae Collection of the Costa Rica Institute of Technology, registered under the code CMiTEC 01. Microselection of fusion products was conducted using an Eppendorf micromanipulator from the Molecular Biology Laboratory of the School of Natural and Exact Sciences, Costa Rica Institute of Technology, San Carlos Campus. Various samples of *C. vulgaris* from the Microalgae Laboratory of the Biotechnology Research Center (School of Biology) at the Costa Rica Institute of Technology, Cartago, were employed.

ELECTROFUSION TESTS

Three tests were conducted to obtain fusion products: enzymatic degradation of the cell wall, alignments (U1-U2), and fusion (Un). These tests established conditions for microalgae cell wall digestion, optimized alignment conditions (U1-U2) by varying the alternating current used, and defined fusion conditions (Un) by determining direct current voltages and the number of pulses for electrofusion. The tests were carried out using an Eppendorf Multiporator.

Enzymatic Degradation of the Cell Wall

Microalgae were treated with two individual enzymatic mixtures to digest their cell walls, facilitating the fusion process. Two enzymatic solutions were

evaluated: 1) 0.06% proteinase K (Sigma Aldrich 3115836001) and 2) 4% cellulase + 2% macerozyme (*Trichoderma reesei* cellulase from Sigma Aldrich C2730 + *Aspergillus niger* pectinase P4716). The enzymes were assessed at intervals of 30 minutes up to 2 hours (30, 60, 90, and 120 minutes) to determine the progress of cell wall degradation and the optimal digestion time. The evaluation involved microscopic observation (Leica DM 750 microscope) with wall measurements over time using Leica LAS EZ software (Leica Microsystem) and analysis with Image J software. Cell counting was performed using a Fuchs Rosenthal counting chamber.

Alignments (U1-U2)

Alignment tests were conducted using purified suspensions of *C. vulgaris* (~1x10^6 microalgae mL^{-1}) in a sodium-free hypoosmolar solution (Eppendorf 940002001). To determine the voltage needed for alignment (U1-U2) of 2 or more microalgae, 200 µL of the microalgae solution was placed in the electrofusion chamber. Various alternating current voltages (7 to 10 V in 1 V increments per test) were evaluated at a constant time of 40 seconds. Descriptive analysis was performed based on the percentages of alignments observed in the microscope field during the tests.

Fusion (Un)

The calculation of the critical electric field current required to induce pore opening (Un) in the *C. vulgaris* membrane was conducted using the following equation (modified from Eppendorf 2007):

$$U_n = \frac{V_c}{0{,}75\ x\ D} xE$$

Where: Un = Required field for fusion (V), Vc = Permeabilization voltage (2 V at 22 ºC), D = Diameter of microalgae (cm), and E = Distance between electrodes (cm). Once the direct current field required for electrofusion was obtained, three pulses were applied at intervals of 30 µs to induce pore opening between the microalgae.

MICROSELECTION OF FUSION PRODUCTS

Microselection of fusion products was performed using the TransferMan® NK2 (Eppendorf) with the CellTram® Air system (Eppendorf) and 60 µm ID

microcapillaries. A total of 20 microselections were conducted per fusion event. Due to the number of samples evaluated, 10 microselections were performed on two different dates, considering this factor as a covariate.

GROWTH OF FUSION STRAINS

Microselected lines of fusion cells obtained under different alternating current voltages for fusion (7, 8, 9, and 10 volts) were cultured in BG11 medium with an initial inoculum size of $1x10^6$ cells mL^{-1} in a volume of 500 mL. Each voltage was set as a treatment with three replicates, and the growth of microalgal lines was evaluated under laboratory conditions, in orbital shaken containers at a temperature between 23 and 28 ºC, illuminated with a light intensity of 60-70 $\mu Em^{-2}s^{-1}$. The growth rate was calculated from 15 mL samplings every two days until day 18 of cultivation for direct cell counting using a Neubauer chamber and dry weight evaluation (g dry weight $L^{-1}d^{-1}$) with 10 mL of culture. Viability and microalgal growth kinetics were determined during the exponential growth phase for each treatment, and the biomass produced per day was calculated.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Enzymatic digestion tests were conducted using a completely randomized design. The treatments analyzed were the two enzymatic solutions (0.06% proteinase K and 4% cellulase + 2% macerozyme) evaluated over time (30, 60, 90, and 120 minutes). Each experimental unit consisted of 500 μL of microalgae with 10 replications distributed over two days (5 replications per day). The amount of cells without cell walls was analyzed through cell counting with a Fuchs Rosenthal chamber.

The electrofusion process study was evaluated using a completely randomized design. Four alignment treatments (U1-U2) were employed, each with 10 replications, five replications per day, with 200 μL of microalgae suspension at a concentration of 1×10^6 microalgae mL^{-1}, as the experimental unit.

In all experiments, replications were distributed over two days (5 replications per day) due to the time required for sample preparation and treatment. This factor was considered a covariate, so an analysis of covariance (ANCOVA) was conducted to determine the main effects of the treatments evaluated. Tukey's mean comparisons were performed to determine if there were significant differences between treatments.

The results of cellular kinetics parameters of *C. vulgaris* fusion lines under the four evaluated treatments were analyzed using ANOVA, and mean comparisons (Tukey, α = 0.05) were performed when statistical differences were determined. The analysis was conducted using the INFOSTAT program (Di Rienzo et al. 2014) through the general linear model (GLM) procedure.

RESULTS

ELECTROFUSION TESTS

During the preparation of microalgae for the fusion process, they underwent enzymatic treatments to permeate the microalgae cell walls, facilitating the subsequent electrofusion process. From the evaluated treatments, it was determined that the enzymatic solution composed of 4% cellulase + 2% macerozyme allowed the highest number of cells with visible changes in the cell wall. Thus, *C. vulgaris* cells reached the highest indices of microalgae with digested walls (2.4 ± 0.01 x 10^5 microalgae mL^{-1}) after 120 minutes of digestion in this enzymatic solution (Fig. 1). Conversely, significant differences were observed in the enzymatic treatment with 0.06% proteinase K, which was not effective in degrading the walls of C. vulgaris.

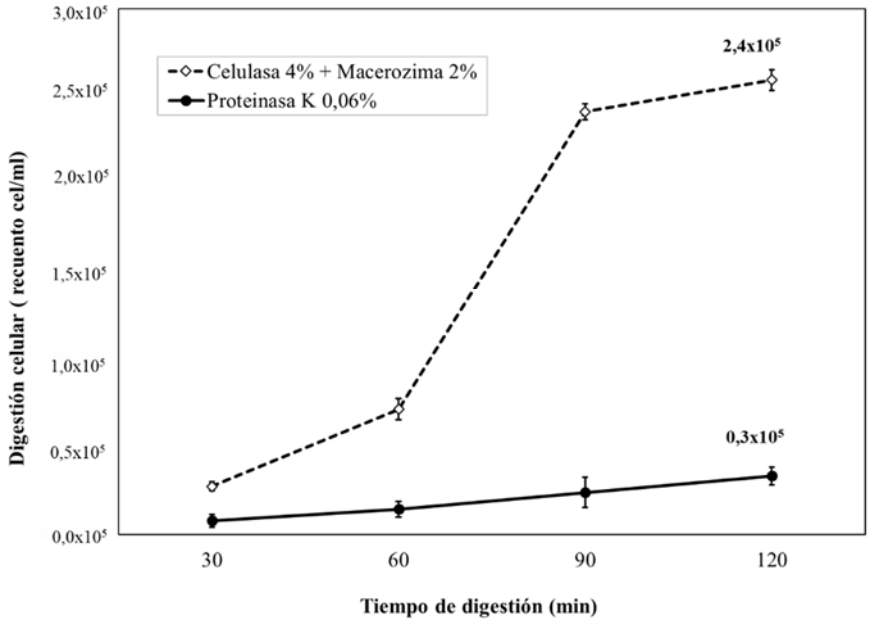


Figure 1. Digestion of the cell wall of *Chlorella vulgaris* in two enzyme mixtures. The bars represent the standard deviation.

ANCOVA showed no significant effect of different treatment dates applied (p=0.5344). However, significant differences (p=0.0001) were observed between the

evaluated treatments and their interaction with digestion time. The digestion times that presented higher indices of digested walls were between 90 – 120 minutes in the cellulase 4% + macerozyme 2% enzymatic solution.

Alignment tests helped establish appropriate voltages for the proper alignment of microalgae during the electrofusion process. Voltages of 9 to 10 V achieved the highest alignment percentages (between 39% and 43%) compared to other evaluated voltages (Fig. 2).

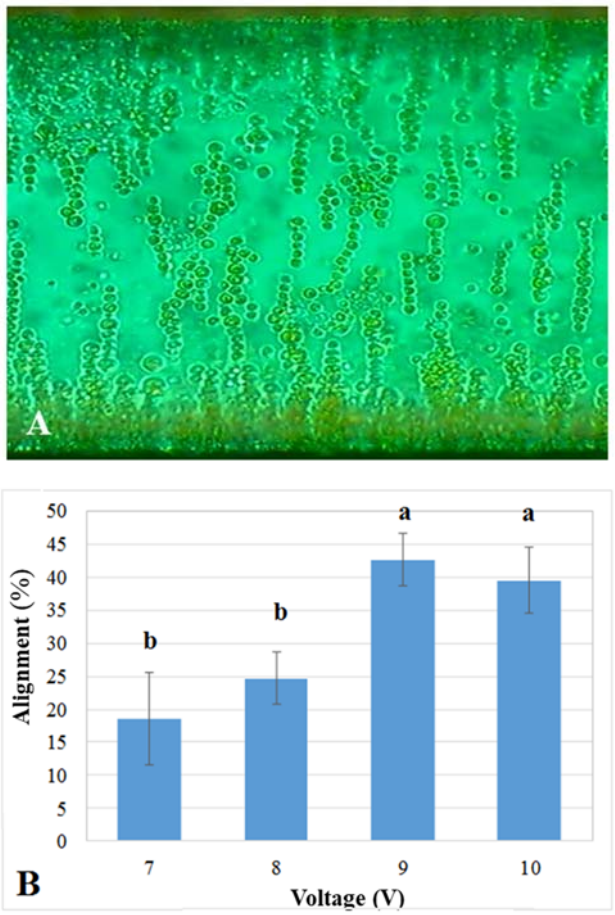


Figure 2. Percentage of alignment of *Chlorella vulgaris* based on different alternating current fields (A. Alignment of *C. vulgaris*, 100X; B. Percentage of alignment. Bars represent the standard deviation. Different letters indicate significant difference, α=0.05)

Again, ANCOVA showed no significant effect between different treatment dates applied (p=0.7688). However, significant differences (p=0.0001) were observed between the applied voltages.

Calculations related to the critical direct current electric field needed to induce pore opening (Un) revealed a significant effect on the required field based on the diameter of the microalgae to be fused (Table 1). Thus, with a permeabilization voltage

(Vc) of 2 V at 25°C, an electrode distance (E) of 0.02 cm, and diameters ranging from 15 to 20 cm (D), it was determined that the voltage required for electrofusion would be ~31 V (DC).

Table 1. Critical direct current electric field required for the electrofusion of *Chlorella vulgaris*.

Diameter of *C. vulgaris* (cm)	**Un (V)**
0.0015	35,6
0.0016	33,3
0.0017	31,4
0.0018	29,6
0.0019	28,1
0.0020	26,7
Average	**30,8**

MICROSELECTION OF FUSION PRODUCTS

Once all parameters for electrofusion (U1=9 V, 30 s; Un=31 V, 30µs; U2=9 V, 60 s) were determined, 20 fusion processes were conducted, followed by microselection of fusion events, recognized by their elongated shape under the microscope when membrane fusion occurred (Fig. 3). This shape remained consistent even when the capillary used for aspiration was moved abruptly.

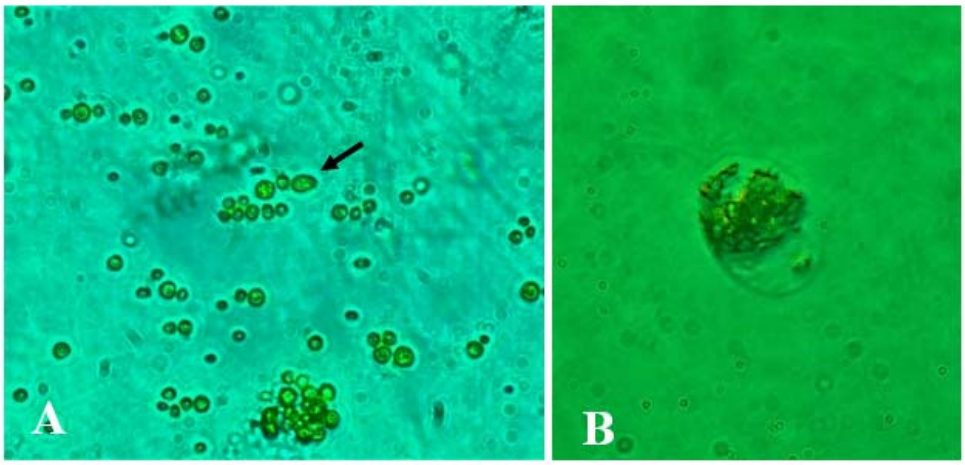


Figure 3. Fusion event observed after electrofusion processes in *Chlorella vulgaris*. (A. After electrofusion, 160X; B. After microselection, 640X). The dark arrow indicates the presence of the fusion event.

GROWTH OF FUSION STRAINS

After the electrofusion process, microselected cells of *Chlorella vulgaris* were able to grow naturally, exhibiting no morphological differences from wild cells, demonstrating the potential of the electrofusion technique to obtain fused protoplasts capable of generating stable offspring.

The graphical analysis of the cellular growth of these fusion events revealed similar microalgal growth for all treatments and a growth curve showing an exponential phase from day 1 to day 10 for the four studied unialgal cultures, followed by a stationary phase until day 16 (Fig. 4A), and finally the death phase. ANOVA analysis determined that the growth rate of the cultures differed statistically based on the voltage used in electrofusion (p=0.002), where the 8 V treatment showed higher growth compared to others (Fig. 4B); biomass productivity in dry weight was statistically different during the exponential growth phase until day 9 (p=0.015), being higher for the 8 and 9-volt treatments (Fig. 4B).

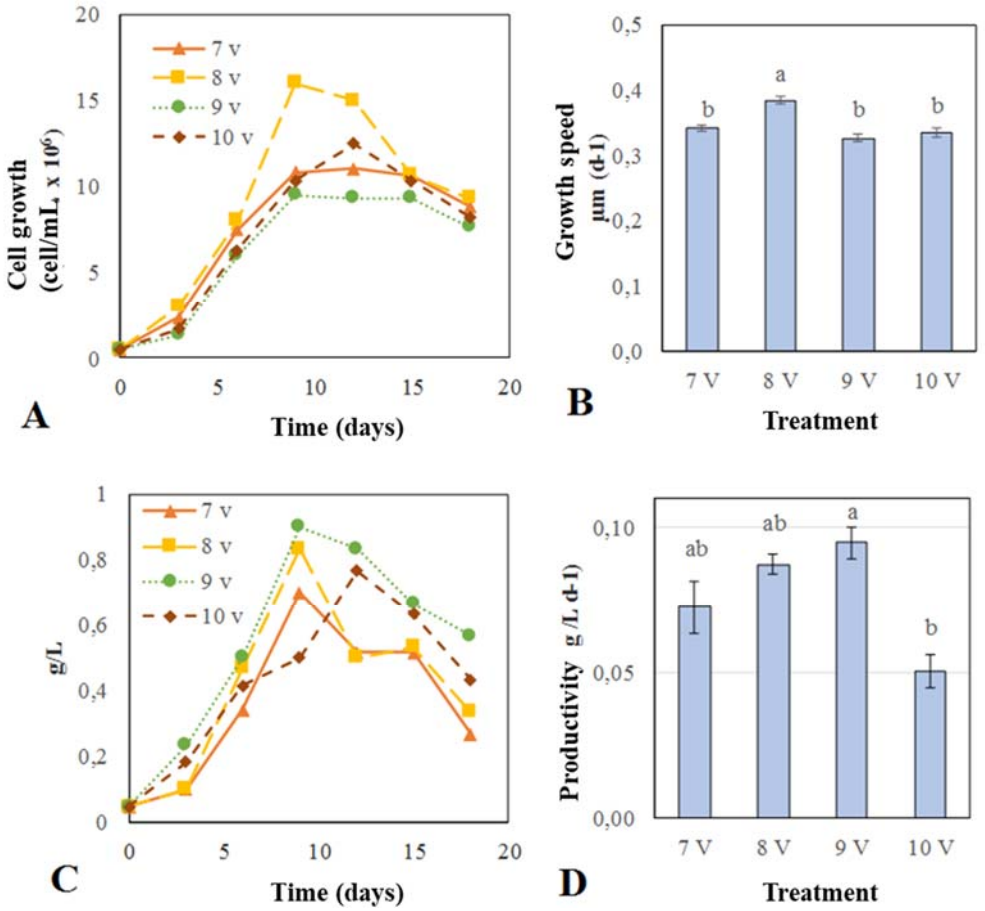


Figure 4. Growth of electrofused unialgal cultures of *C. vulgaris* in BG-11 medium according to alignment voltages. (A. Cell growth in cells mL^{-1}, x106; B. Growth rate in exponential phase; C. Dry weight in grams per liter; D. Dry biomass productivity at the end of the exponential phase on day 9. Bars represent the standard deviation. Different letters indicate significant difference, α=0.05).

DISCUSSION

By applying cellulase, macerozyme, and pectinase enzymes to promote the digestion of the cell wall to facilitate electrofusion between *C. vulgaris* cells and obtain protoplasts, it was observed that 4% cellulase in conjunction with 2% macerozyme favored the degradation of the cell wall. This process was primarily due to the release of glucose resulting from cell wall degradation (Rosen et al., 1985). Although proteinase K has been used for microalgae wall degradation (Gerken et al., 2013, Abomohra et al., 2016), our study did not show a positive effect in obtaining *C. vulgaris* protoplasts; in fact, it could have inhibitory effects on its growth (Gerken et al., 2013).

During alignment, microalgae with digested walls migrated towards the poles caused by the alternating current induced by the Eppendorf multiporator (Montero and Jiménez 2015), inducing the formation of aligned chains of microalgae in the induced electric field (Fig. 2A). This was facilitated by the low conductivity of the medium using the hypoosmolar solution, preventing the voltage from exceeding the critical electric field that induces cell death, thus avoiding the destruction of the generated protoplasts (Jaroszeski et al., 1994). According to Van Wert and Saunders (1992), the time and the amount of alignment are directly related to the alternating current voltage applied to each cell species being worked with, highlighting the importance of determining these conditions for the studied *C. vulgaris* species (Van Wert and Saunders 1992). In our study, voltages from 9 to 10 V allowed higher alignment indices in shorter periods (30 s). When applying direct current shocks, the critical direct current electric field used (31 V) allowed the formation of pores on the surfaces of contact between two or more microalgae (Tung, 1999, Eppendorf, 2007), which could facilitate the subsequent mixing of cytoplasmic contents during post-alignment (U2). This facilitated the formation of heterokaryons due to the mixing of both cellular contents (Montero-Carmona and Jiménez 2009).

The technique of cell fusion has become popular in the development of genetic studies worldwide. More recently, the highly efficient method of using milli- and microsecond-applied electric fields to induce cell-to-cell fusion, cell-to-particle fusion, or to induce the entry of DNA or other compounds into cells is being implemented (Rems et al. 2013; Luengo et al. 2015). However, studies on the procedure and cell survival are necessary since supplying electric voltages for long periods to cell membranes causes the loss of their integrity, disrupting molecular transport and inducing the ability of microalgae to maintain their homogeneity, leading to inactivation (Qin et al. 2014).

To follow up on the fusion events generated in our facilities, a microselection method was implemented using the TransferMan® NK2 equipment (Eppendorf) and a CellTram® Air system (Eppendorf) with 60 μm ID microcapillaries, successfully

separating the visualized fusion events from the rest of the non-fused microalgae (Fröhlich and König 2000). This accelerated the process of selection and production of microalgal cultures from fused cells (Duong et al. 2012). The use of microselection as a method for isolating and cultivating prokaryotic organisms was developed by Ishøy et al. (2006). A new and highly efficient microselection method, similar to that reported in this study, is currently being implemented for the isolation and study of denitrifying bacteria, with high efficiency in terms of time and obtaining lines from individual cells (Ashida et al., 2010).

Our results indicate that the applied electric currents did not affect the survival of microalgae, and they were able to reproduce until reaching cellular concentrations equal to or even higher than those observed in non-electrofused strains. According to Wong et al. (2017), in their study of the cellular kinetics of *C. vulgaris* cultivation in different media, this species reaches a maximum growth rate of 0.279 ± 0.001 d^{-1} and a biomass productivity of 0.114 g/L d^{-1}. The growth capacity of our electrofused lines would allow the application of this technique to fuse protoplasts from microalgal lines with different potentials, for example, to increase the production of carotenoids, lipids, and resistance to environmental conditions by fusing wild strains with highly bioactive compound-producing strains. Previous studies also demonstrate the possibility of obtaining fusion cells from different species (Kusumaningrum, and Zainuri, 2015), and the protocol obtained in this study should be tested in the fusion of different microalgal strain-lines.

CONCLUSION

This work allowed the establishment of an efficient electrofusion method for *Chlorella vulgaris*, through the application of 4% cellulase + 2% macerozyme (for 90 min) to digest the cell wall and facilitate electrofusion. The application of the established electrofusion parameters (U1= 9 V, 30 s; Un = 31 V, 30 μs; U2 = 9 V, 60 s) and the microselection of fusion events resulted in overall growth rates for all fusion events of up to 0.8 g/L at day 9.

ACKNOWLEDGMENTS

A heartfelt thanks to the Vice Presidency for Research and Extension at the Costa Rican Institute of Technology for funding the project "Establishment of unialgal cultures through microselection and microalgae injection for the production of renewable energy sources" (Functional Center 2151057), from which this publication originated.

REFERENCES

Abomohra AE-F, El-Sheekh M, Hanelt D. Protoplast fusion and genetic recombination between *Ochromonas danica* (Chrysophyta) and *Haematococcus pluvialis* (Chlorophyta). Phycologia. 2016;55(1):65-71. DOI: https://doi.org/10.2216/15-88.1

Almeida HN, Calixto GQ, Chagas BM, Melo DM, Resende FM, Melo MA. Characterization and pyrolysis of *Chlorella vulgaris* and *Arthrospira platensis*: potential of bio-oil and chemical production by Py-GC/MS analysis. Environ Sci Pollut Res Int. 2017;24(16):14142-14150. DOI: https://doi.org/10.1007/s11356-017-9009-2

Ashida, N., Ishii, S., Hayano, S., Tago, K., Tsuji, T., Yoshimura, Y., Otsuka, S., and Senoo, K. Isolation of functional single cells from environments using a micromanipulator: Application to study denitrifying bacteria. Appl. Microbiol. Biotechnol., 2010; 85, 1211-1217. DOI: https://doi.org/10.1007/s00253-009-2330-z

Braun E, Aach H. Enzymatic degradation of the cell wall of *Chlorella*. Planta. 1975;126(2):181-185. DOI: https://doi.org/10.1007/BF00380622

Chow K-C, Tung W. Electrotransformation of *Chlorella vulgaris*. Plant Cell Rep. 1999;18(9):778-780. DOI: https://doi.org/10.1007/s002990050660

Di Rienzo J, Balzarini M, Gonzalez L, Casanoves F, Tablada M, Robledo C. InfoStat/L software estadístico versión 2014. Universidad Nacional de Córdoba (FCA-UNC)[Internet]. 2010.

Duong VT, Li Y, Nowak E, Schenk PM. Microalgae isolation and selection for prospective biodiesel production. Energies. 2012;5(6):1835-1849. DOI: https://doi.org/10.3390/en5061835

Eppendorf. 2007. Multiporator®: Basic Applications Manual for Electrofusion. Hamburgo, AL, Eppendorf Publications. p. 4 – 22.

Fan J, Ning K, Zeng X, Luo Y, Wang D, Hu J, et al. Genomic foundation of starch-to-lipid switch in Oleaginous *Chlorella* spp. Plant Phys. 2015;169(4):2444-2461. DOI: https://doi.org/10.1104/pp.15.01174

Fröhlich J, König H. New techniques for isolation of single prokaryotic cells. FEMS Microbol Rev 2000;24(5):567-572. DOI: 10.1111/j.1574-6976.2000.tb00558.x

Gerken HG, Donohoe B, Knoshaug EP. Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. Planta. 2013;237(1):239-253. DOI: https://doi.org/10.1021/ba-1977-0160.ch008

Göbel E, Aach H. Studies of *Chlorella* protoplasts. II. Isolation and fusion of protoplasts from *Chlorella saccharophila* (Krüger) Nadson strain 211-1a. Plant Sci. 1985;39(3):213-218. DOI: https://doi.org/10.1016/0168-9452(85)90177-3

Gong Y, Hu H, Gao Y, Xu X, Gao H. Microalgae as platforms for production of recombinant proteins and valuable compounds: progress and prospects. J Ind Microbiol Biotechnol. 2011;38(12):1879-1890. DOI: https://doi.org/10.1007/s10295-011-1032

Ishøy T, Kvist T, Westermann P, Ahring BK. An improved method for single cell isolation of prokaryotes from meso-, thermo-and hyperthermophilic environments using micromanipulation. Appl Microbiol Biotechnol. 2006;69(5):510-514. DOI: https://doi.org/10.1007/s00253-005-0014-x

Jaroszeski MI, Gilbert R, Fallon PG, Heller R. Mechanically facilitated cell-cell electrofusion. Biophys J 1994;67(4):1574-1581. DOI: https://doi.org/10.1016/S0006-3495(94)80630-7

Kumar KS, Dahms HU, Won EJ, Lee JS, Shin KH. Microalgae–A promising tool for heavy metal remediation. Ecotoxicol Environ Saf. 2015;113:329-352. DOI: https://doi.org/10.1016/j.ecoenv.2014.12.019

Kusumaningrum, H. P., & Zainuri, M. Optimization and Stability of Total Pigments Production of Fusan from Protoplast Fusion of Microalgae *Dunaliella* and *Chlorella in vivo*: Attempts on Production of Sustainable Aquaculture Natural Food. Int. J. Mar. Aquat. Res. Conserv. Co-Exist. 2015; 1(1), 1-5.

Luengo E, Martínez Jm, Coustets M, Álvarez I, Teissié J, Rols M-P, et al. A comparative study on the effects of millisecond-and microsecond-pulsed electric field treatments on the permeabilization and extraction of pigments from *Chlorella vulgaris*. J Membr Biol 2015;248(5):883-891. DOI: https://doi.org/10.1007/s00232-015-9796-7

Montero-Carmona W, Jiménez VM. Identificación y selección de híbridos somáticos obtenidos mediante fusión de protoplastos. Biotecnología vegetal. 2009;9(2).

Montero-Carmona, W.; Jiménez, V. M., Vanilla Protoplasts: Isolation and Electrofusion. In: Rakshit A, editor. Emerging Innovations in Agriculture: From Theory to Practice. Athens Institute for Education and Research, 2015. p. 15–29.

Qin S, Timoshkin IV, Maclean M, Wilson MP, Macgregor SJ, Given MJ, et al. Pulsed electric field treatment of microalgae: Inactivation tendencies and energy consumption. IEEE T Plasma Sci. 2014;42(10):3191-3196. DOI: https://doi.org/10.1109/TPS.2014.2317522

Rems L, Ušaj M, Kandušer M, Reberšek M, Miklavčič D, Pucihar G. Cell electrofusion using nanosecond electric pulses. Sci Rep-Uk. 2013; 3: 3382. DOI: https://doi.org/10.1038/srep03382

Rivera AL, Gómez-Lim M, Fernández F, Loske A. Genetic transformation of cells using physical methods. Genet Syndr Gene Ther. 2014;5(4):237-242. DOI: https://doi.org/10.4172/2157-7412.1000237.

Rosen BH, Berliner Md, Petro MJ. Protoplast induction in *Chlorella pyrenoidosa*. Plant Sci. 1985;41(1):23-30. DOI: https://doi.org/10.1016/0168-9452(85)90061-5

Specht E, Miyake-Stoner S, Mayfield S. Micro-algae come of age as a platform for recombinant protein production. Biotechnology letters. 2010;32(10):1373-1383. DOI: https://doi.org/10.1007/s10529-010-0326-5

Talebi AF, Tohidfar M, Tabatabaei M, Bagheri A, Mohsenpor M, Mohtashami Sk. Genetic manipulation, a feasible tool to enhance unique characteristic of *Chlorella vulgaris* as a feedstock for biodiesel production. Molecular biology reports. 2013;40(7):4421-4428. DOI: https://doi.org/10.1007/s11033-013-2532-4

Van Wert SL, Saunders JA. Electrofusion and electroporation of plants. Plant physiology. 1992;99(2):365-367. DOI: https://doi.org/10.1104/pp.99.2.365

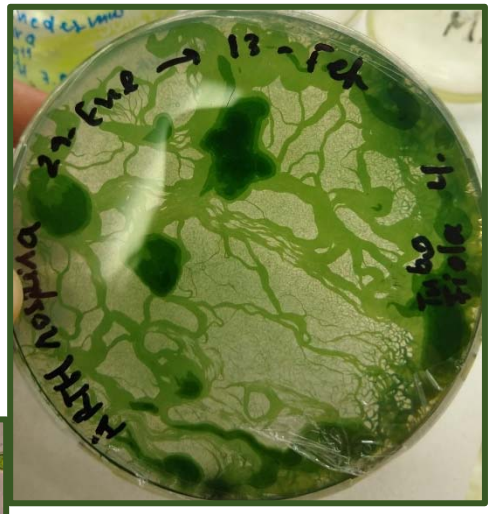
Wang L, Min M, Li Y, Chen P, Chen Y, Liu Y, et al. Cultivation of green algae *Chlorella* sp. in different wastewaters from municipal wastewater treatment plant. Applied biochemistry and biotechnology. 2010;162(4):1174-1186. DOI: https://doi.org/10.1007/s12010-009-8866-7

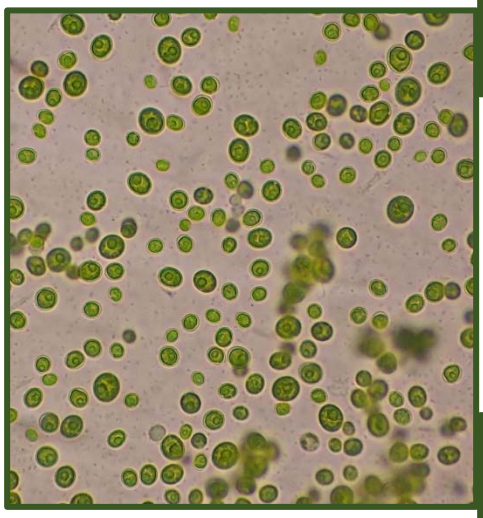
Wong, Y., Ho, Y. H., Ho, K. C., Leung, H. M., & Yung, K. K. L. Growth Medium Screening for *Chlorella vulgaris* Growth and Lipid Production. J. Aquac. Mar. Biol. 2017;6(1), 00143.

Yamada T, Sakaguchi K. Comparative studies on *Chlorella* cell walls: Induction of protoplast formation. Archives of Microbiology. 1982;132(1):10-13. DOI: https://doi.org/10.1007/BF00690809

Yamada T, Sakaguchi K. Protoplast induction in *Chlorella* species. Agricultural and Biological Chemistry. 1981;45(8):1905-1909. DOI: https://doi.org/10.1016/0304-4211(77)90099-2

Yang J, Cao J, Xing G, Yuan H. Lipid production combined with biosorption and bioaccumulation of cadmium, copper, manganese and zinc by oleaginous microalgae *Chlorella minutissima* UTEX2341. Bioresource technology. 2015; 175:537-544. DOI: https://doi.org/10.1016/j.biortech.2014.10.124.







RENUWAL

PROGRAMA IBEROAMERICANO
CYTED
CIENCIA Y TECNOLOGÍA PARA EL DESARROLLO