

## PRODUCCIÓN DE LÍPIDOS A PARTIR DE MICROALGAS PSICROFÍLICAS, PRESENTES EN LOS GLACIARES ANTÁRTICOS PARA LA SÍNTESIS DE BIODIESEL

### PRODUCTION OF LIPIDS FROM PSYCHROPHILIC MICROALGAE PRESENT IN ANTARCTIC GLACIERS FOR THE SYNTHESIS OF BIOFUEL

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#### RESUMEN

Las microalgas han tenido un impacto negativo en la calidad sensorial en general. Las microalgas psicrófilas viven en ambientes de frío extremo. Además, su crecimiento aumenta porque tienen en su estructura enzimas que solo se adaptan a temperaturas por debajo de 0 °C. Por este motivo, el Laboratorio de Química Sostenible de la Universidad Central del Ecuador, junto con el Instituto Antártico del Ecuador (INAE), realizó una expedición en Greenwich, Roberts, Dee, Barrientos y Antarctic Towers donde se recolectaron varias colecciones de microalgas, donde se analizaron 15 muestras de Greenwich Island y Roberts durante 21 días en diferentes niveles de temperatura, de los géneros *Chlorella* sp, *Chlorococcum* sp y *Stichococcus* sp. Posteriormente, se realizó el aislamiento en placas Petri para obtener cultivos de monoalgal. Cada uno de los géneros aislados se masificó en un volumen de 5 ml hasta alcanzar un volumen de 250 ml en medio M1 modificado a una temperatura de 4 °C y 24 °C, 5000 lux y un fotoperíodo de 12:12 horas. El método de Bligh & Dyer se utilizó para la extracción de lípidos. Los valores de la concentración de lípidos mostraron que el género *Chlorella* sp tiene la concentración más alta con un valor de 0,2802 mg/ml a 4 °C y un valor de 2.6704 mg/ml a 24 °C, el día 22 de su fase exponencial en comparación con los géneros *Chlorococcum* y *Stichococcus* sp.

**Palabras claves:** *Chlorella* sp, *Chlorococcum*, *Stichococcus* sp., Antártica, Lípidos, método Bligh & Dyer.

#### ABSTRACT

Microalgae had a negative impact on the overall sensory quality. Psychrophilic microalgae live in extremely cold environments, their growth increases because they have enzymes in their structure that only adapt to temperatures below 0 °C. For this reason, the Sustainable Chemistry Laboratory of the Central University of Ecuador, together with the Ecuadorian Antarctic Institute (INAE), made an expedition in the Greenwich, Roberts, Dee, Barrientos and Antarctic Towers where several microalgae consortia were collected, where 15 samples from Greenwich Island and Roberts were analyzed at 21 days at different temperatures, from which the genera *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp. Subsequently, isolation was made in Petri dishes to obtain monoalgal cultures. Each of the isolated genera was massified in a volume of 5 mL until reaching a volume of 250 mL in modified M1 medium at a temperature of 4 °C and 24 °C, 5000 lux and a photoperiod of 12:12 hours. The Bligh & Dyer method was used for the extraction of lipids. The values of the lipid concentration showed that the genus *Chlorella* sp is the highest concentration with a value of 0.2802 mg / mL at 4 °C and a value of 2.6704 mg / mL at 24 °C on the 22nd day of its exponential phase in comparison with the genera *Chlorococcum* and *Stichococcus* sp.

**Keywords:** *Chlorella* sp, *Chlorococcum*, *Stichococcus* sp., Antarctica, Lipids, Bligh & Dyer method

## 1. INTRODUCTION

Psychrophilic microalgae are photosynthetic organisms that belong to the plant kingdom and adapt to temperatures of  $-5^{\circ}\text{C}$  (Martínez, 2010). In Ecuador, studies of psychrophilic microorganisms have been conducted in the Chimborazo Glaciers (Correa, 2013). The psychrophilic microalgae collected in the Greenwich, Roberts, Dee, Barrientos and Torre islands of the Antarctic Archipelago have survived to the present, according to data from the Pedro Vicente Maldonado Antarctic Station, the type of genera identified in the microalgae consortium has increased by 2. in 2013 to 6 in 2014. There are 35 samples collected in total, of which 11 belong to Greenwich Island and correspond to the largest number of samples collected containing psychrophilic algae so far. The collections that also have a high abundance belong to the Greenwich Island with 5 genera. The psychrophilic microalgae that have less survival belong to Dee Island (Ecuador Antarctic, 2014). The Sustainable Chemistry Laboratory of the Central University of Ecuador is a government entity that aims to obtain different genera of microalgae from the samples collected, as well as the isolation of the genera: *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp, to carry out processes at a pilot scale to subsequently extract lipids and synthesize biodiesel. The research focuses on the field and laboratory area to study the essential oil extracted from psychrophilic microalgae to make way for the synthesis of biofuel as an alternative source of diesel, as well

as the isolation and massification of surviving microalgae (Flores, 2013). The obtaining of biofuel is extracted from lipids of microalgae collected at low temperatures belonging to the Chlorophyta, Bacillariophyta and Euglenophyta division (Amaro, Guedes, & Malcata, 2011). The most prevalent genera are *Haematococcus* sp., *Chlorella* sp., *Stichococcus* sp., *Diatoma* sp., *Navicula* sp. and *Filamentosa* sp, are characterized by being present in the three years of sampling (Molina, 2015). The marine and freshwater species that contain the highest amount of lipids for the production of biodiesel are *Dunaliella* with 116 mg / L / day and *Nannochloris* with 76.5 mg / L / day, as well as the genus *Chlorella* sp with 50 mg / L / day (MALGAS, 2013). In our country, no psychrophilic microalgae genera have been reported for the synthesis of biodiesel (Flores, 2013). The open systems for obtaining microalgae were developed between 1935 and 1940 due to the scarcity of food. The application was made on an industrial scale with the purpose of obtaining an alternative source of proteins of vegetable and animal origin to replace food for human consumption (Colorado, Moreno, & Pérez, 2013). The first microalgae were massified to obtain lipids during the Second World War in Germany, the studies were carried out by German scientists with the purpose of purifying contaminated waters (MALGAS, 2013). In 1953, microalgae cultures are studied for the use of photosynthetic gas exchangers used in space travel and as sources of microbial proteins (BEAM, 2013). In 1980 the cultivation of microalgae

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is produced for the improvement of the environment, transforming the organic samples and sediments from the wastewater into biomass and renewed waters for later use in biofertilizers and water for irrigation. In 1982 at world level, oil increased value, giving alternative ideas such as the use of microalgae as a source of solar energy (Colorado, Moreno, & Pérez, 2013). From 2013 to 2015, samples of microalgae were collected in the islands near the Pedro Vicente Maldonado Scientific Station, climate diversity is an important factor in the synthesis of fatty acids to use them as an alternative source of energy. The sample collected at Punta Figueroa on Greenwich Island in Antarctica has guaranteed the best consortium of microalgae in the production of effective oils such as behemoth, myristic, stearic, palmitic, linoleidic, oleic, palmitoleic, arachidic and linoleidal. The genus of microalgae and the temperature from which the sample was obtained is important for obtaining lipids in dry weight, as well as the extraction methods used (Flores, 2013).

Currently, the European Union has implemented ideas to develop energy as biodiesel from microalgae consortia with the purpose of reducing derivatives from oil sources and the emissions produced by gases during the greenhouse effect (Wackett, 2008). Among the genera of microalgae identified with higher lipid productivity are *Chlorella* sp and *Chlorococcum* sp (Flores, 2013). But there are major drawbacks that researchers face with the use of microalgae: the presence of high concentrations of

biomass means that more energy is required, and the moderate amount of biofuel produced by microalgae on a pilot scale (AINEnergía, 2015). The cryophyllous microorganisms commonly known as psychrophilic inhabit extremely cold environments, their growth increases because they have enzymes in their structure that only adapt to temperatures below 0 ° C. The cell membrane is made up of fats that flow to the point of freezing (Barreiro & Sandoval, 2006). Among the psychrophilic Antarctic microalgae is *Chlorella* sp, which shows rapid growth in cell cultures (Chinnasamy & Bhatnagar, 2010). Its shape is spherical, green because it contains high levels of chlorophyll (Kanno & Kazie, 2005). Therefore, researchers have opted for studies with psychrophilic microalgae, which could represent a safe alternative for the production of lipids. Among the psychrophilic Antarctic microalgae investigated are mainly: *Pandorina* sp, *Chlorella* sp, *Stichococcus* sp, *Chlamydomona* sp and *Chlorococcum* sp (Molina, 2015). Other results obtained in microalgae such as *Scenedesmus ovalternus*, *Chlorella vulgaris*, *Nannochloropsis* sp and *Isochysis* sp, have determined the production of lipids with a growth of 32.7 mg / L / day, obtaining a large amount of biodiesel. These results allow us to conclude that some genera of microalgae could be useful for the production of lipids (Bérmudez, 2012).

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## 2. MATERIALS AND METHODS

### 2.1. Collection of samples.

The samples were awarded by the Sustainable Chemistry Laboratory of the Central University of Ecuador as part of the project of the Ecuadorian Antarctic Institute (INAE). These were taken from Greenwich Island and Roberts Island located in Antarctica, later moved to Ecuador in different test tubes with their respective covers.

### 2.2. Adaptation process

Several samples of biomass were taken from the original tubes, where consortiums 15IGa2 with coordinates 35 ° 59 '36' 'S and 48 ° 27' 27 ' ' O, consortium 15IGc1 with coordinates 35 ° 53 '30' 'S and 76 ° 43 '17' 'O and the 15IRe1 consortium with coordinates 35 ° 43' 40 ' ' and 72 ° 13 '27' 'O were adapted to different temperatures in the laboratory after going through a 21-day adaptation process in function of the modified culture medium M1 as shown in Table 1.

**Table 1.** Adaptation of the genres of consortium 15IGa2, 15IGc1 and 15IRe1 at 21 days.

Consortium	Original Sample	Adaptation 4 ° C	Adaptation 24 ° C
15IGa2	<i>Haematococcus sp.</i>	<i>Chlorella sp.</i> <i>Chlorococcum sp</i>	They did not survive
	<i>Chlorococcum sp.</i>		
	<i>Chlorella sp.</i>		
	<i>Stichococcus sp.</i>		
	<i>Diatoma sp.</i>		
15IGc1	<i>Chlorella sp.</i>	<i>Chlorella sp.</i> <i>Stichococcus sp.</i> <i>Chlorococcum sp</i>	They did not survive
	<i>Stichococcus sp.</i>		
	<i>Chlorococcum sp.</i>		
	<i>Chlorella sp.</i>		
15IRe1	<i>Haematococcus sp.</i>	<i>Chlorococcum sp.</i> <i>Chlorella sp.</i>	<i>Chlorococcum sp.</i> <i>Chlorella sp.</i> <i>Stichococcus sp.</i>
	<i>Chlorococcum sp.</i>		
	<i>Chlorella sp.</i>		
	<i>Stichococcus sp.</i>		

### 2.3. Isolation in solid culture medium.

This technique was used to separate the three selected microalgae genera

after the adaptation process in this case were *Chlorella sp* belonging to consortium 15IGa2, *Chlorococcum sp* belonging to consortium 15IGc1 and *Stichococcus sp* belonging to

consortium 15IRe1. Agar was added to the modified M1 medium and 40 mL were distributed in three Petri dishes, then two drops of each consortium were inoculated in each of the boxes and spread with a sterile bacteriological loop. The respective lid was placed and the box was covered with parafilm paper, the Petri boxes were inverted and placed at 24 ° C with a 24 hour photoperiod, illumination of 4500 lux for two weeks. The microalgae were observed in the inverted microscope.

#### 2.4. Preparation of culture medium

Three test tubes were used with 5 mL of modified M1 medium and a small amount of each microalga isolated from its respective Petri box was

placed with a 24 hour photoperiod at 24 ° C with 4500 lux illumination, placing a stopper on the part top of the tube so that it has aeration for two weeks. The culture medium used for the microalgal growth was the modified M1 medium.

#### 2.5. Modified culture medium M1

The medium was prepared in three different bottles according to the amount of volume required, the volume of distilled water was measured with a 1000 mL pipette, then the three bottles were autoclaved for 90 minutes at a pressure of 15 PSI and a temperature of 121 ° C. The composition of the modified M1 medium is shown in Table 2 (Salas, 2015).

**Table 2.** Composition of modified culture medium M1 for 1000 mL (Salas, 2015)

STOCK	CONCENTRATION
<b>Stock I</b>	<b>(g / L)</b>
KH <sub>2</sub> PO <sub>4</sub>	0.3
K <sub>2</sub> HPO <sub>4</sub>	0.18
<b>Stock II</b>	<b>(g / L)</b>
FeCl <sub>3</sub> .6H <sub>2</sub> O	0.002
<b>Stock III</b>	<b>(g / L)</b>
NaCl	0.1
CaCl <sub>2</sub>	0.02
MgSO <sub>4</sub>	2.65
FeSO <sub>4</sub>	0.002
KNO <sub>3</sub>	0.7025
KCl	0.30
ZnSO <sub>4</sub>	0.0007
H <sub>3</sub> BO <sub>3</sub>	0.034
EDTA	0.03
MnSO <sub>4</sub>	0.003
CoCl <sub>2</sub>	0.02 mg
CuSO <sub>4</sub>	0.3
Na <sub>2</sub> Mo	0.04 mg

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## **2.6. Conditions culture microalgae psychrophilic Antarctic**

Once growth was obtained in each test tube, it was massed in 18 bottles of 250 mL. The genera *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp were inoculated into the modified M1 medium in three bottles of 250 mL per each gender at temperatures of 4 ° C and 24 ° C. A system was added to each of the bottles. Aeration of 0.5 L / min, a fuse glass with a diameter of 0.6 mm was attached to a hose, this system was connected to a 5000 Power Pump of 60 Hz, 5 W and 110 V with 2 outputs. Lighting was installed using lamps at 5000 lux with a photoperiod of 12:12 hours. The jars are monitored every day. A cell count was performed using the Neubauer chamber every 3 days. A data record was kept for 28 days to obtain values in the graph of absorbance vs lipid concentration, using the Excel 2008 program.

## **2.7. Bligh & Dyer extraction method**

Five mL of each vial with its respective microalga at different temperatures in 18 test tubes was placed, placing aluminum foil on top to avoid contamination, placed in the oven at 80 ° C in a period of 48 hours. After this time elapsed, 3 mL of a mixture of chloroform-methanol solvents (1: 2) was added. The test tubes (Tube 1) were sonicated in 3 cycles of 15 minutes by adding distilled water and ice. The tubes were incubated at 4 ° C for 24 hours protecting them from the light with aluminum foil. The next day

the tubes were sonicated in 3 cycles of 15 minutes adding distilled water and ice. They were centrifuged at 4700 rpm for 20 minutes at 24 ° C, then the extracts were recovered with a Pasteur pipette, to transfer the supernatant to new 15 mL tubes (Tube 2). 1.5 mL of a chloroform methanol (1: 2) solvent mixture was added to the residual biomass of (Tube 1), this step is done to extract more dry biomass lipids from the microalgae. It was again centrifuged at 4700 rpm for 20 minutes at 24 ° C recovering more extract from (Tube 1) to place it in (Tube 2).

2 mL of distilled water from (Tube 2) containing the extract was added and vortexed. The excess water was removed with a Pasteur pipette, and the last centrifugation was performed at 4700 rpm for 10 minutes at 24 ° C to separate the lower phase formed by chloroform and lipids. Then 1 mL of chloroform was added to further separate the lower phase, a Pasteur pipette was introduced with great care, air was bubbled to the bottom of the tube. The phase of chloroform and lipids was passed from (Tube 2) to (Tube 3), and how much volume was taken was measured. The aqueous phase was washed with 1 mL of chloroform, mixed in the vortex and centrifuged at 4700 rpm for 10 minutes at 24 ° C, the lower phase was recovered and placed in (Tube 3). It was left in a water bath overnight with shaking at 37 ° C without a lid. The next day, after the chloroform was evaporated, 2 mL of concentrated sulfuric acid were added. The tubes were sealed with aluminum foil in order to avoid contamination, a blank was prepared (Tube with sulfuric acid). The tubes

were heated in an oven at 200 ° C for 15 minutes without lids. The tubes were placed in the refrigerator at 4 ° C to cool, 3 mL of distilled water was added. The tubes were sonicated for 15 minutes and mixed with vortex until the sample was homogeneous

and without residues of organic matter. The samples were transferred to the cells and the absorbance was read at 375 nm in the spectrophotometer. The materials used are shown in Table 3.

**Table 3.** Materials used in the Bligh & Dyer Method

Equipment	Reagents	Materials
	Test tubes	
Refrigerator	Aluminum foil	Distilled water
Sonicador	Glass bottles	Ice
Centrifuge	100 mL test tube	Chloroform
Vortex	Pipettes	Methanol
Digital balance	Petri boxes	Sulfuric acid
	Stove	

### 2.8. Quantification of lipids extracted by spectrophotometry

The amount of lipids extracted from the genera *Chlorella* sp, *Chlorococcum* sp *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp was obtained in the spectrophotometer. To perform the quantification, all the steps mentioned above were developed in the Bligh & Dyer extraction method to measure the absorbance at 375 nm. The concentration of lipids obtained from each gender at different temperature was calculated by the following equation:

$$[Lipids] = \left( \frac{Ab - 0.0126}{2.8311} \right) \times \frac{3.5}{V_{ext}} \quad (\text{Eq. 1})$$

Where: Ab (Absorbance), Vext (Volume extracted from the sample).

The concentration of lipids indicated the lipid production in time. These data were calculated through the SPSS 15.0 program The equation of the line was obtained with experiments previously carried out at the Central University of Ecuador.

$$y = 2.831 + 0.0126 \quad (\text{Eq. 2})$$

$$R^2 = 0.999$$

### 2.9. Determination of the growth curve of *Stichococcus* sp.

To obtain the dry weight of the genus *Stichococcus* sp. in the stationary phase to determine the growth curve, first the test tubes were weighed on the analytical balance before placing the volume of each bottle, then 5 mL of each bottle was placed at a

temperature of 4 °C and 24 °C In the 6 test tubes measured above, aluminum foil was used in the upper part to avoid contamination in the sample, then the tubes were placed in the oven at 80 °C in a period of 48 hours. Once the dry biomass was obtained inside each tube, it was reweighed in the analytical balance, the results were taken in a register and analyzed using the Excel 2008 program. Through the analysis of ANOVA and Duncan's Test, the factor temperature and gender in the production of lipids for the synthesis of biofuel was determined through the SPSS 15.0 program.

### 3. RESULTS AND DISCUSSIONS

#### 3.1. *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp.

##### Massification

Microalgae samples obtained from the 15IGa2, 15IGc1 and 15IRe1 consortiums of Greenwich Island and Roberts Island of the Antarctic Islands

were conserved in 3 test tubes, with modified M1 medium, in a 24 hour photoperiod at a temperature of 24 °C with aeration and illuminance of 4500 lux. After two weeks of isolation, the microalgae isolated in their respective Petri box were observed, and the genera *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp. in the modified M1 medium with three repetitions of each in bottles of 250 mL at 4 °C and 24 °C in a photoperiod of 12:12 hours, with moderate aeration and with illuminance of 5000 lux.

##### Analysis of cell growth at different temperatures

The values indicated in Table 4 and Table 5 in relation to cell growth at the temperature of 4 °C, indicated that in three replications (A, B and C), the average number of *Chlorella* sp cells per mL in the exponential phase on day 22 was 285'000 000 cel / mL, compared to the genus *Chlorococcum* sp which was 95'100 000 cel / mL, both microalgae were at the same temperature, phase, illumination, photoperiod and aeration.

**Table 4.** Average cell growth of the genus *Chlorella* sp at 4 °C

<i>Chlorella</i> / M1				
DAYS	A	B	C	AVERAGE
0	0	0	0	0
4	1,40E + 06	1.38E + 06	1.39E + 06	1.39E + 06
11	3,10E + 06	3.09E + 06	3,11E + 06	3,10E + 06
15	2,80E + 07	2,90E + 07	2,84E + 07	2,85E + 07
18	1,00E + 08	1.04E + 08	1.08E + 08	1.04E + 08
22	2,90E + 08	2.81E + 08	2,85E + 08	2,85E + 08
25	2,60E + 08	2,55E + 08	2.67E + 08	2.61E + 08

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**Table 5.** Average cell growth of the genus *Chlorococcum* sp at 4 °C

<i>Chlorococcum</i> / mL				
DAYS	A	B	C	AVERAGE
0	0	0	0	0
4	4.67E + 05	4.60E + 05	4.63E + 05	4.63E + 05
11	1.03E + 06	1.03E + 06	1.04E + 06	1.03E + 06
15	9.33E + 06	9,67E + 06	9,47E + 06	9,49E + 06
18	3,33E + 07	3,47E + 07	3,60E + 07	3,47E + 07
22	9,67E + 07	9,37E + 07	9,50E + 07	9.51E + 07
25	8.67E + 07	8,50E + 07	8.90E + 07	8.69E + 07

The three repeats (A, B and C) of the genus *Stichococcus* sp at a temperature of 4 °C indicated an average value of cells per gram on day 22 of its exponential phase of 0.88 cel / g, in

this case performed the dry weight count, due to the fact that a cell count cannot be executed in the Neubauer Chamber due to its morphology. The values are shown in Table 6.

**Table 6.** Average cell growth of the genus *Stichococcus* sp at 4 °C

<i>Stichococcus</i> / g				
DAYS	A	B	C	AVERAGE
0	0	0	0	0
4	0.02	0.02	0.02	0.02
11	0.16	0.17	0.16	0.16
15	0.38	0.36	0.36	0.37
18	0.65	0.68	0.68	0.67
22	0.88	0.90	0.88	0.88
25	0.74	0.78	0.78	0.76

The data indicated in Table 7 and Table 8 in relation to cell growth at 24 °C, showed that in three replications (D, E and F) the average cell number of *Chlorella* sp per mL in the

exponential phase in the day 22 was 604,000,000 cel / mL compared to the genus *Chlorococcum* sp which was 201,000,000 cel / mL, both genera were in equal conditions.

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**Table 7.** Average cell growth of the genus *Chlorella* sp at 24 °C

<i>Chlorella</i> / mL				
DAYS	D	E	F	AVERAGE
0	0	0	0	0
4	1,35E + 06	1.36E + 06	1.34E + 06	1,35E + 06
11	1.38E + 08	1,48E + 08	1,52E + 08	1,46E + 08
15	3.53E + 08	3.05E + 08	3,11E + 08	3,23E + 08
18	4.70E + 08	4,75E + 08	4,80E + 08	4,75E + 08
22	6,00E + 08	6.02E + 08	6,10E + 08	6.04E + 08
25	5,26E + 08	4.78E + 08	5,18E + 08	5.07E + 08

**Table 8.** Average cell growth of the genus *Chlorococcum* sp at 24 °C

<i>Chlorococcum</i> / mL				
DAYS	D	E	F	AVERAGE
0	0	0	0	0
4	4.50E + 05	4.53E + 05	4,47E + 05	4.50E + 05
11	4,60E + 07	4.94E + 07	5.05E + 07	4,86E + 07
15	1,18E + 08	1.02E + 08	1.04E + 08	1.08E + 08
18	1,57E + 08	1.58E + 08	1,60E + 08	1.58E + 08
22	2,00E + 08	2.01E + 08	2.03E + 08	2.01E + 08
25	1,75E + 08	1,59E + 08	1.73E + 08	1,69E + 08

The three repetitions (D, E and F) of the genus *Stichococcus* sp at 4 °C presented an average value of cells for each gram on day 22 of its exponential

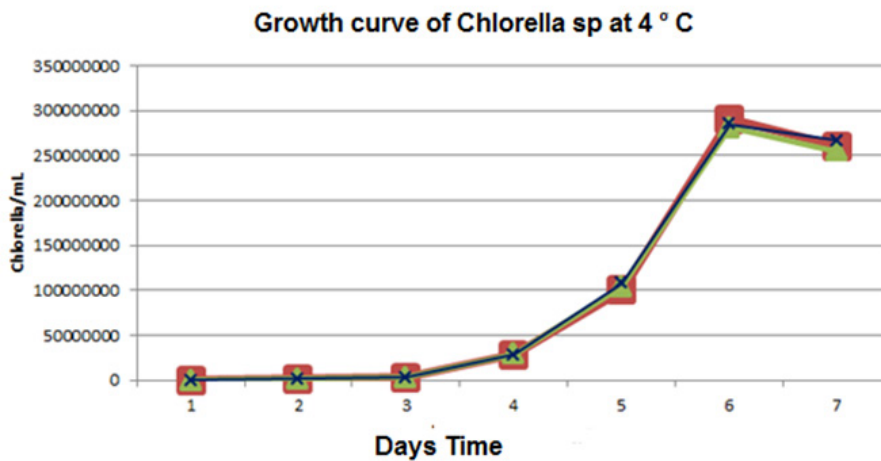
phase of 1.18 cel/g, the cell count was performed by dry weight. The values are indicated in Table 9.

**Table 9.** Average cell growth of the genus *Stichococcus* sp at 4 °C

<i>Stichococcus</i> / g				
DAYS	D	E	F	AVERAGE
0	0	0	0	0
4	0.13	0.15	0.15	0.14
eleven	0.30	0.30	0.29	0.30
fifteen	0.60	0.63	0.63	0.62
18	0.80	0.83	0.80	0.81
22	1,15	1,18	1.20	1,18
25	0.85	0.78	0.78	0.80

The genera *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp grew in the modified M1 medium that contained several macroelements and microelements to contribute to microalgal growth (Salas, 2015).

Figure 1 and Figure 2 show that *Chlorella* sp microalgae had greater cell growth at 28 days in its three repetitions at 4 °C and 24 °C in relation to the genus *Chlorococcum* sp indicated in Figure 3 and Figure 4.



**Figure 1.-** Growth curve of *Chlorella* sp cultivated in M1 modified in 3 repetitions at 4 °C.

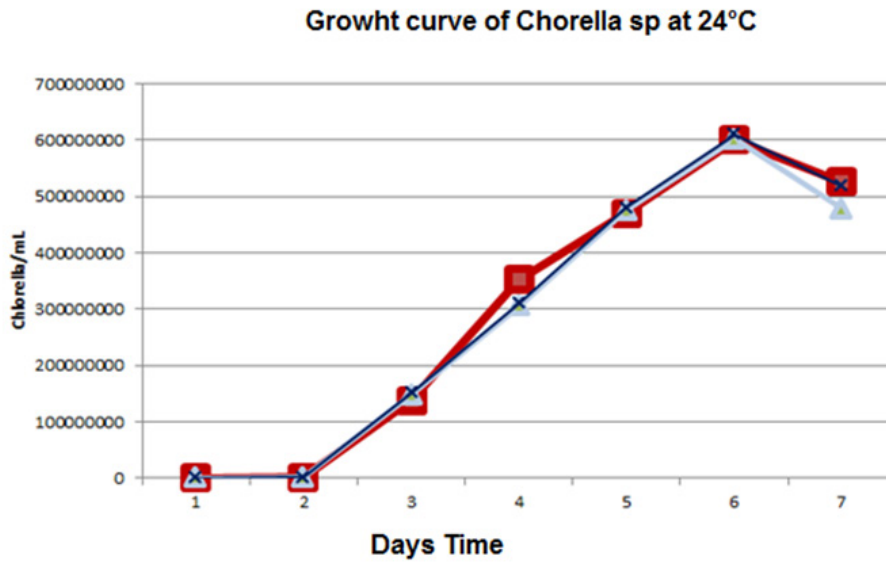


Figure 2.- Growth of curve *Chorella* sp cultivated in M1 modified in 3 repetitions at 4 °C.

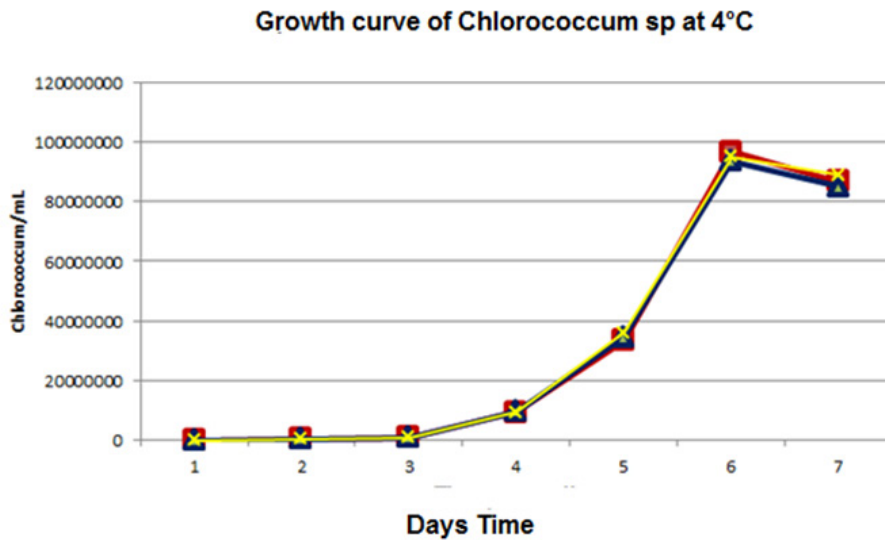


Figure 3.- Growth curve of *Chlorococcum* sp cultivated in M1 modified in 3 repetitions at 4 °C.

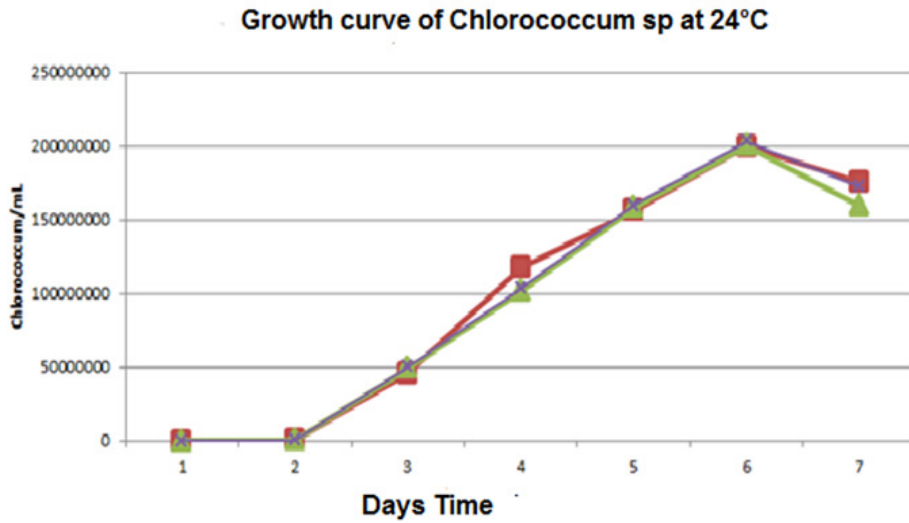


Figure 4.- Growth curve of Chlorococcum sp cultivated in M1 modified in 3 repetitions at 24 °C.

Figure 5 and Figure 6 show the cell growth of Stichococcus sp at 28 days, with three repetitions at different

temperatures. This microalga was under the same conditions as Chlorella sp and Chlorococcum sp.

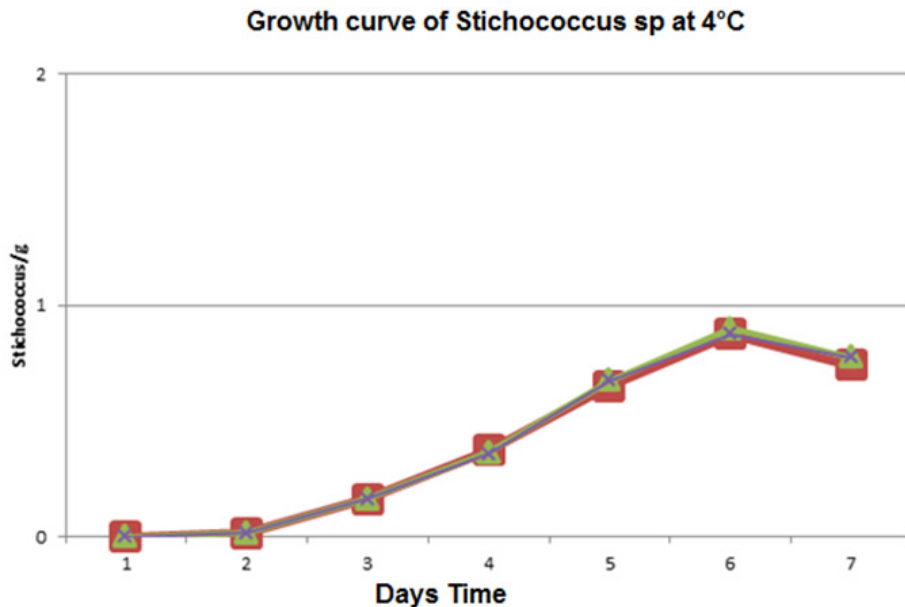
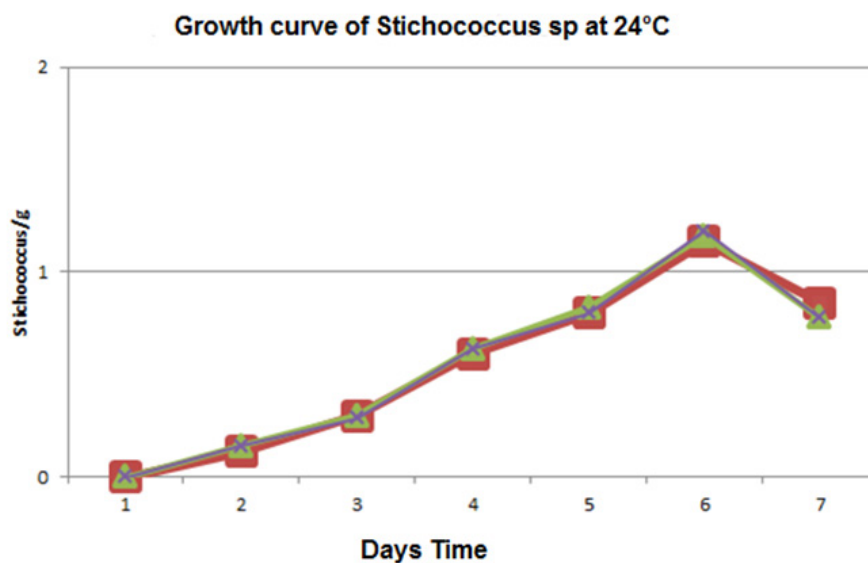


Figure 5.- Growth curve of Stichococcus sp cultivated in M1 modified in 3 repetitions at 4 °C.



**Figure 6.-** Growth curve of *Stichococcus* sp cultivated in M1 modified in 3 repetitions at 24 °C.

The genus *Chlorella* sp showed greater cellular growth until day 22 of its exponential phase in comparison with the genera *Chlorococcum* sp and *Stichococcus* sp, because it has a better capacity of absorption of CO<sub>2</sub>, which is why it captured all the nutrients contained in the culture medium used which in this case was the modified M1 (Khotimchenko & Yakolevka, 2005). The time in which the microalgal cells were generated is optimal for the production of lipids and subsequently sintering biofuel and several metabolites. It should also be noted that the modified culture medium M1 favors the growth of the microalga due to the nitrogen and phosphorus components in its composition (Fernández, Sánchez, & Molina, 2001). The cell growth of *Chlorella* sp can be identified in the color according to the light that the microalga receives. At a temperature of 4 °C they showed

a dark green coloration, this is given by the cyanelles that they have inside. As the bottles preserved at 24 °C they took a light green tone, after 7 days they suffer a deterioration by the photo-oxidative effect since the light and the external oxygen act as secondary factors on the microalga, so that more volume of the modified medium M1 was placed, this causes the cells to acquire again the nutrients that were administered at the beginning and thus be able to assimilate components that have not yet been captured by the genus (*Chisti*, 2007). The modified M1 medium was not used by the *Chlorococcum* sp and *Stichococcus* sp for cell growth. One of the factors that influences is the composition that the medium possesses, for this reason other different culture media must be tested until finding the appropriate one in future projects to carry out cell growth and later the extraction of lipids



(Jaramillo, 2011). The BBM culture medium allows the development of a large number of cells of *Chlorococcum* sp and *Stichococcus* sp although it presents a small amount of nitrogen in its composition (Kuma, 2010).

#### Measurement of extracted lipids

The data obtained in Table 10 in relation to the extraction of lipids of the genera *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp, indicated that the lipid concentration of the genus *Chlorella* sp at a temperature of 4 °C on day 22 of the exponential phase it was 0.2802 mg / mL in comparison with

the genus *Chlorococcum* sp which presented a concentration of 0.0922 mg / mL and the genus *Stichococcus* sp which was 0.0685 mg / mL. All genera were at the same conditions of aeration, light and photoperiod. Likewise, the concentration of lipids extracted at a temperature of 24 °C was greater in the genus *Chlorella* sp on day 22 of the exponential phase with a value of 2.6702 mg / mL, while in the genus *Chlorococcum* sp and *Stichococcus* sp, the concentration was 0.8890 mg / mL and 0.5595 mg / mL respectively, as shown in Table 11 .

**Table 10.** Lipid concentration of microalgae genera at 4 °C

Days	Lipids <i>Chlorella</i> sp [mg / mL]	Lipids <i>Chlorococcum</i> sp [mg / mL]	Lipid <i>Stichococcus</i> sp [mg / mL]
0	0	0	0
4	0,1167	0,0380	0.0177
11	0,1682	0,0549	0,0445
15	0.1961	0.0642	0,0519
18	0,2130	0,0698	0.0551
22	0.2802	0,0922	0,0685
25	0.2403	0,0789	0,0587

**Table 11.** Lipid concentration of the microalgae genera at 24 °C

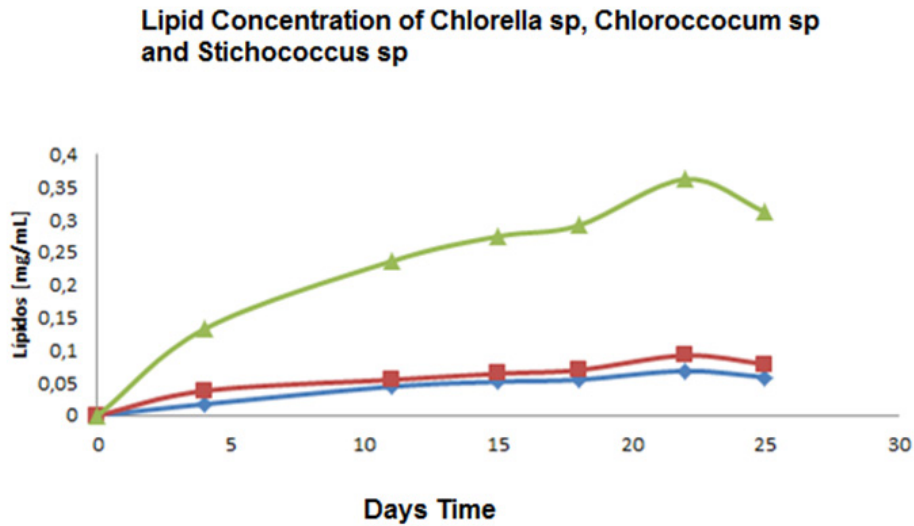
Days	Lipids <i>Chlorella</i> sp [mg / mL]	Lipids <i>Chlorococcum</i> sp [mg / mL]	Lipid <i>Stichococcus</i> sp [mg / mL]
0	0	0	0
4	0,0740	0,0238	0,0180
11	0,2331	0,0765	0.0653
15	0,2765	0,0910	0,0671
18	1,2562	0.4175	0,1785
22	2,6704	0.8890	0.5595
25	1,7097	0,5687	0.4309

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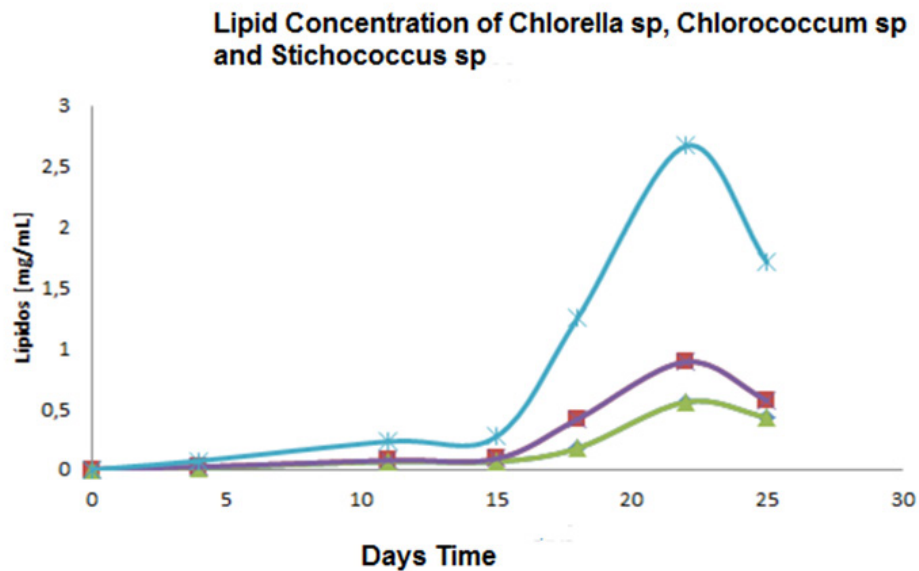
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Figure 7 and Figure 8 show the average of lipid concentrations at 28 days, with

three repetitions at a temperature of 4 °C and 24 °C.



**Figure 7.-** Average concentration of Lipids of Chlorella sp, Chlorococcum sp and Stichococcus sp at 4°C.



**Figure 8.-** Average concentration of Lipids of Chlorella sp, Chlorococcum sp and Stichococcus sp at 24°C.

*Chlorella* sp adapted at 4 °C showed higher concentration of lipids compared to *Chlorococcum* sp and *Stichococcus* sp. The lower temperatures increased the level of unsaturation and the light intensity of 5000 lux favored the microalgae in the accumulation of triglycerides with a high saturation level (Anderson & Katja, 2003). In addition, the genus *Chlorella* sp at 24 °C showed higher lipid production due to the capture of solar energy and CO<sub>2</sub> from the environment. This microalgae is one of the genera with the highest percentage of lipids, the *Chlorella emersonii* species produces 63% in comparison with the species *Chlorococcum oleofaciens* that presents 44.3% (Faife, Otero, & Álvarez, 2012). *Chlorella* sp presents higher productivity of lipids at different temperatures due to the high level of triglycerides that contains, in addition to the genetic constitution, the light intensity, pH, salinity, minerals that were factors that influenced the lipid production (Lee, Lewis, & Ahsman, 2009). In all three genera studied there is a variation in the lipid content. The genera *Chlorococcum* sp and *Stichococcus* sp showed very low lipid levels because their cell growth could occur under unfavorable conditions, there were not enough stress conditions and they did not assimilate all the nutrients of the culture medium administered at both temperatures (Liang, Dong, Miao, & Dai, 2006). The optimal culture medium for these microalgae genera is the BBM or Bristol because it contains a stock solution of macronutrients, a stock of micronutrients and a stock of traces. In addition, the pH of the

medium, the concentrations of each of the stocks, the nitrogen composition, as well as the external factors for the development of cellular growth of microalgae should be taken into consideration (MALGAS, 2013). The genera *Chlorella* sp and *Chlorococcum* sp and *Stichococcus* sp presented lipid production at different temperatures, these results were obtained thanks to the Bligh & Dyer method, which allows to extract a large amount of fatty acids using methanol and chloroform as solvents, without the use of heat (Salas, 2015) The analysis of variance was performed for the cell growth of *Chlorella* sp and *Chlorococcum* sp at different temperatures, where these hypotheses were verified. The analysis of variance was carried out for the lipid concentration of the genera *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp at different temperatures, where these hypotheses were verified.

Ho: Microalgae gender effect = 0

Ha: Microalga genre effect  $\neq$  0

Ho: Temperature effect = 0

Ha: Temperature effect  $\neq$  0

By means of the analysis of variance or ANOVA in the SPSS 15.0 program as shown in Table 12, it was verified that the meanings given by the microalgae and temperature gender factor, related to the significance of 0.05 are lower, so that proceeds to reject the null hypotheses of equality in the factors. For this reason, it was analyzed that the *Chlorella* sp microalgae had a higher lipid concentration at different temperatures.

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**Table 12.** Analysis of ANOVA or variance of the genera *Chlorella sp*, *Chlorococcum sp* *Stichococcus sp* at different temperatures.

Variance analysis					
Source	Sum of Squares Type III	gl	Half quadratic	F	P
Model	4,054	5	0.811	3,935	0.0001
Genus	1,647	2	0.823	3,996	0.0001
Temperature	1,646	1	1,646	7,987	0.0001
Error	6,181	30	0.206		
Total	13,917	36			

Note: To accept the null hypothesis  $P > 0.05$ . F = Ratio of mean squares, P = significance

The Duncan Test was analyzed through the SPSS 15.0 program to check the genus of microalgae that had the highest lipid production taking into consideration the temperature at which each one of them was. In Table 13 the analysis of the gender factor was carried out, resulting in the *Chlorella sp* microalga indicating a

mean value of 0.619, the value is high so it was grouped in a subset, while the genus *Chlorococcum sp* showed a mean value of 0.205 and the genus *Stichococcus sp* had a mean value of 0.134, presenting low levels for this reason were grouped in the same subset.

**Table 13.** ADuncan test of the genera *Chlorella sp*, *Chlorococcum sp* *Stichococcus sp* at different temperatures.

Duncan Alpha test = 0.05			
Error: 0.206		gl: 30	
Gender	Socks	N	Subset
<i>Chlorella sp</i>	0.619	12	1
<i>Chlorococcum sp</i>	0.205	12	2
<i>Stichococcus sp</i>	0,134	12	2

#### 4. CONCLUSIONS

All psychrophilic microalgae present in glaciers in Antarctica produce lipids for the synthesis of biofuel, the genus *Chlorella sp* being the highest concentration with a value of 0.2802 mg / mL at a temperature of 4 °C

and a value of 2, 6704 mg / mL at a temperature of 24 °C on day 22 of its exponential phase. The analysis of the lipid composition shows that the temperature and composition of the modified M1 culture medium are important for the adaptation of the microalgal cells, when these are at

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temperatures of 4 °C and 24 °C, of which the samples adapted to room temperature showed greater cell growth. All samples of microalgae obtained from the 15IGa2, 15IGc1 and 15IRE1 consortiums of Greenwich Island and Roberts Island of the Antarctic Islands were initially massed at a volume of 5 mL, from which the genera *Chlorella* sp, *Chlorococcum* sp and *Stichococcus* sp were isolated. until reaching a volume of 250 mL, in a 24-hour photoperiod with aeration and illuminance of 4500 lux. The extraction of lipids was carried out from the Antarctic psychrophilic microalgae using the Bligh & Dyer method for the synthesis of biofuel. The genus *Chlorella* sp generates more lipids at a temperature of 24 °C, and in a small amount at a temperature of 4 °C. The genera *Chlorococcum* sp and *Stichococcus* sp generated a low amount of lipids at different temperatures, taking into account that the lipid concentration is low, it is not ruled out that other factors have influenced the production of lipids.

Among the psychrophilic Antarctic microalgae is *Chlorella* sp, which shows rapid growth in cell cultures (Chinnasamy & Bhatnagar, 2010). Its shape is spherical, green because it contains high levels of chlorophyll (Kanno & Kazie, 2005).

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