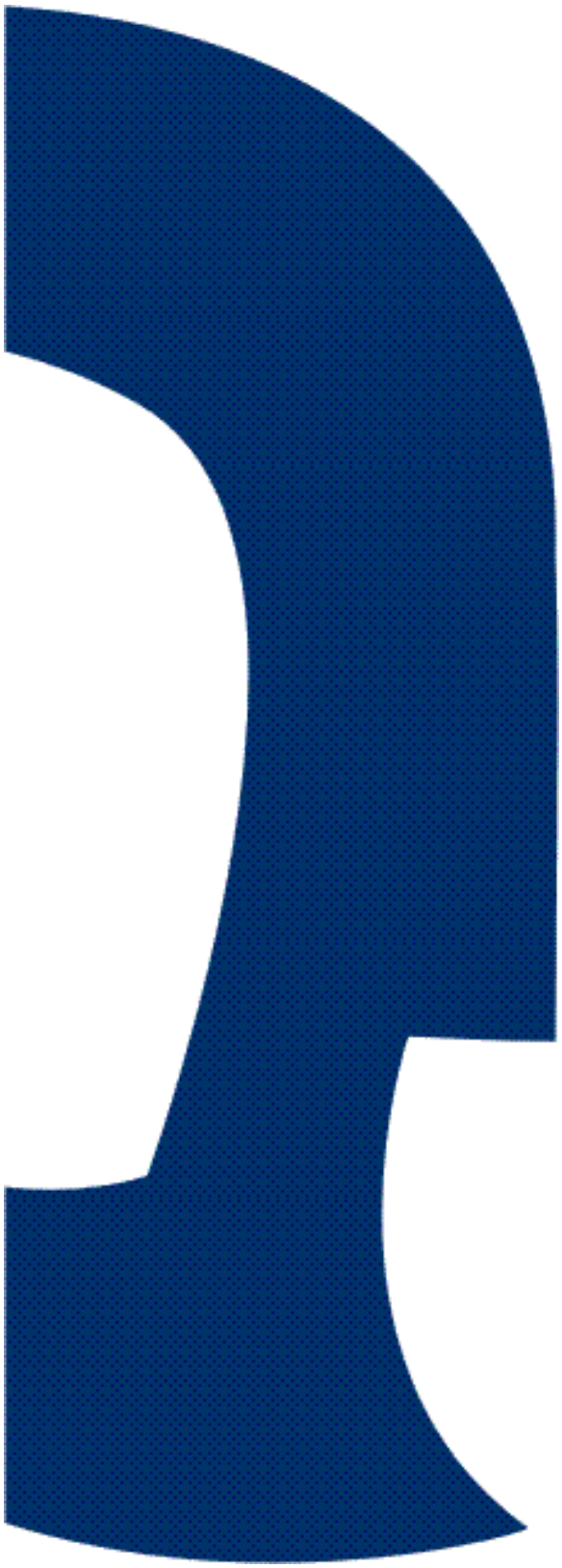
FACULTAD DE CIENCIAS DEL MAR

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA



1.

Microalgae and cyanobacteria selection for agriculture applications (Project SABANA). Verification of chlorophyll fluorescence methodology for biomass yield determination

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2. Index

A B S T R A C T

The present study was developed within the SABANA (Sustainable Algae Biorefinery for Agriculture aNd Aquaculture) Project which is based on looking for species of microalgae that supply three specific function from the synthesis of secondary metabolites, namely biopesticides, biostimulants in agriculture and aquaculture. In this regard 18 microalgae species were tested under laboratory conditions with controlled photoperiod, temperature and CO_2 adition. Only 4 of the 18 cultures started could be used to perform growth curves and characterization. Biomass harvested in the study was analyzed in further investigations for the extraction of metabolites.
Moreover, the verification of a recent method to calculate primary productivity was carried out. The principle of this method is to establish a linear relationship between the chlorophyll a fluorescence parameter (Fo) and chlorophyll a (Chl a) content *via* extraction. Chl a pigment extraction is a slow process. Using this simple, cheap and efficient method it was possible to verify the linear relationship for different microalgae species and morphology.

Keywords: microalgae, SABANA Project, chlorophyll fluorescence, Chl a, growth curves, calibration curves, optical density, Fo - Basal fluorescence, biomass production

RESUMEN

El estudio ha sido desarrollado dentro del Proyecto SABANA (Sustainable Algae Biorefinery for Agriculture aNd Aquaculture) en el que se están seleccionando especies de microalgas y cianobacterias que contengan metabolitos secundarios para el empleo como estimulante agrícola, biopesticidas y en acuicultura aplicando el concepto de biorefinería. Se han utilizado 18 especies distintas de algas en cámara de cultivo con condiciones controladas de temperatura, fotoperiodo e inyección de CO_2. De estas 18 especies se utilizaron 4 que sirvieron para caracterizar el crecimiento, y la producción de biomasa para los análisis y bioensayos posteriores.
El segundo de los objetivos del trabajo fue la verificación de un método reciente para el cálculo de la producción, basado en establecer una relación linear entre la fluorescencia de la clorofila (Fo) y otros parámetros como el contenido en Chl a o el contaje celular de los cultivos. Esta relación ha sido verificada con distintos grupos taxonómicos y

características morfológicas de manera que, siendo un método simple, rápido y barato, sirva para estimar datos de producción de biomasa en los cultivos controlados.

Palabras clave: microalgas, Proyecto SABANA, fluorescencia de la clorofila, Clorofila a, curvas de crecimiento, densidad óptica, Fo – fluorescencia basal, producción de biomasa

3. 1. Introduction

Microalgae are unicelular and photosynthetic microorganisms, ranging from 0.2 to 2 μm up to filamentous forms with higher sizes (Ravindran et al., 2016). They include both, prokaryotic (cyanophyceae) and eukaryotic organisms. Microalgae are able to grow in a broad range of environments, including freshwater, seawater, wastewaters and extreme conditions of temperature, pH and salinity. This allows them to colonise a broad range of ecological niches.

Microalgae are photosynthetic organisms that play a key role in aquatic ecosystems. Approximately 40% of global photosynthesis is carried out by these organisms (Moreno-Garrido, 2008). Microalgal metabolism reacts to changes in the external environment by changing its intracelular environment and synthesizing secondary metabolites that have chemical structures unprecedented in the terrestrial organisms displaying interesting biological activity (Shimizu, 2003). These compounds have received much attention due to their potential in a vast field of applications and situated microalgae as a powerful biotechnological platform for the production of a broad range of algae-derived products (Wolf et al., 2015).

This study is focused on the developments for the European H2020 Project SABANA (Sustainable Algae Biorefinery for Agriculture aNd Aquaculture), which aims to obtain secondary metabolites for three main applications; biopesticides and biostimulants for agriculture and feed for aquaculture applications. Fulfilling the objectives of the project, which is the development and demonstration of a microalgae-based integrated biorefinery for large-scale production of valuable compounds for agricultural and aquaculture uses, with the premise of sustainability.

For that reason, only marine water in addition to wastewater containing nutrients and flue gases from other industries will be used. When using freshwater, the costs of producing microalgae is high (>5 €/kg of dry mass), but using sewage and flue gases costs can be reduced by one order of magnitude (<0,5 €/kg). The final goal of this project is to achieve an environmentally and economically sustainable zero-waste process at a demonstration scale up to 5 ha.

A major concern of projects related to the Spanish Bank of Algae is to improve the biology of the cultivation process and the management of the cultures. By doing so, the quality and safety of the produced biomass is ensured, allowing the selection of strains with higher capacity to be produced at large scale and demonstrating higher content of biostimulants, biopesticides or feed additive.

Moreover, microalgae are the largest contributor to primary production in water environment and allow for quantification measuring the Chl a content of microalgae is required. Methodology of extracting chlorophyll a (Chl a) is slow and requires appropriate material. Recently a new method was published to measure primary production (Chen et al., 2017). It is based on establishing a linear relationship between Fo (Fluorescence basic parameter of Chl a) and Chl a content. As measuring Fo is simple, rapid and inexpensive the validation of this method by using different morphological strains of the assayed species seemed an objective with interest.

4. 2. Objectives

The main objectives of the present work were focused on:
(1) Selection and characterization of microalgae (including cyanobacteria) strains from marine environment at laboratory scale, according to the established developments provided by the SABANA Project.

(2) Verification of a new method to determine productivity values on different microalgae strains (included on the working list) with different morphological characteristics based on chlorophyll fluorescence parameters (Fo).

5. 3. Material and methods

3.1. Organism and growth conditions

Experiments were developed with marine microalgae and cyanobacteria from the collection of the Spanish Bank of Algae (Table 1). These microalgae seawater strains were used based on possibilities for three different applications; Cyanobacteria for biopesticides and eucarioticmicroalgae for biostimulants and aquaculture.

Table 1

Microalgae species used in this study and applications: green for biopesticides, yellow for biostimulants and blue for aquaculture.

BEA Code	Strain name	Taxonomic group	Medium	Bioprospected
BEA 1211B	*Leptolyngbya sp*	Cyanobacteria	F/2	Spain, Canary Islands, Fuerteventura, Aguas Verdes beach
BEA 1267B	*Leptolyngbya sp*	Cyanobacteria	F/2	Not available
BEA 1412B	*Lyngbya sp*	Cyanobacteria	F/2	Spain, Las Palmas, Gran Canaria, Viera y Clavijo Botanical Garden, little pot filled with rain water
BEA 1269B	*Cyanobacteria sp*	Cyanobacteria	F/2	Spain, Las Palmas, Fuerteventura. Aguas Verdes Beach; Green balls on the rocks at the intertidal zone
BEA 1247B	*Pseudophormidium sp*	Cyanobacteria	F/2	Spain, Las Palmas, Gran Canaria. Natural Pools- Castillo del Romeral; Scraped from the stair of the pool
BEA 1303B	*Chroococcidiopsis sp*	Cyanobacteria	F/2	Spain, Las Palmas, Fuerteventura. Aguas Verdes Beach; Biofilm at the intertidal zone
BEA 1302B	*Lyngbya sp*	Cyanobacteria	F/2	Spain, Las Palmas, Fuerteventura. Aguas Verdes Beach; Biofilm at the intertidal zone.
BEA 1183B	*Daesonia sp*	Chlorophyceae	F/2	Spain, Las Palmas,

BEA 0745B	*Scenedesmus bajacaliforniacus*	Chlorophyceae	F/2	Gran Canaria. Depuranat (Wastewater Treatment lagoon) Campus Universitario de Tafira; Biofilm on wood board around the water tanks Spain, Vizcaya. Nervión river; brackish water
BEA 0069	*Halochlorella rubescens*	Chlorophyceae	F/2	Spain, Las Palmas, Fuerteventura. Lobos-Islote de Lobos Natural Park; Sandy crust lava flows.
BEA 0505B	*Entomoneis sp*	Bacillariophyceae	F/2	Spain, Santa Cruz de Tenerife, La Palma. Nueva Beach-Volcanes de Teneguía Natural Monument
BEA 0937B	*Euglena cantabrica*	Euglenophyceae	BBM	Spain, Las Palmas, Gran Canaria, Charca de Maspalomas (plain water)
BEA 0666B	*Spyrogyra sp*	Zygnematophyceae	BG11	Spain, Las Palmas, Gran Canaria. Azuaje Ravine-Azuaje Special Nature Reserve ; Duck tank
BEA 0046	*Parachlorella sp*	Trebouxiophyceae	F/2	Cape Verde, Maio, Maio. Porto Ingles Saltworks; Salt crust
BEA 0499B	*Navicula sp*	Bacillariophyceae	F/2	Spain, Santa Cruz de Tenerife, La Palma. Nueva Beach-Volcanes de Teneguía Natural Monument
BEA 0313B	*Sarcinochrysis marina*	Pelagophyceae	F/2	Spain, Las Palmas, Gran Canaria. La Aldea de San Nicolas; Harbour. Squeezed macroalgae
BEA 0595B	*Rebecca sp*	Haptophyceae	F/2	Spain, Las Palmas, Fuerteventura. El Cotillo; Near the lighthouse. Taken from sandy beach, moist aliquot

BEA 0860B	*Ochrospora verrucosa*	Haptophyceae	F/2	Spain, Las Palmas, Gran Canaria. Castillo de San Cristóbal; Intertidal rock pool near the castle

3.2. Culture medium

Because most of the algae were marine species, the main culture medium used was F/2 (Guillard, 1975) (Table 2). The base of this medium was seawater bi-filtered first with 1 μm and then by 0,2 μm filter. BG11 (Stanier et al., 1971) (Table 3) medium and BBM (Bischoft and Bold, 1963) (Table 4) were also used for two freshwater algae used as control, namely BEA 0937B and BEA 0666B. For these mediums miliQ water was used and the pH was adjusted to 6,6 for BBM and 7,4 for BG11.

3.3. Culture conditions

Growth experiments were carried out in the culture chamber with a temperature of 25 ± 2°C, the photoperiod was set to 18 hours of light and 6 hours of darkness (18:6 L:D) and the aereation provided CO_2 1 min pulses every hour.

The culture was started with 25 ml of the strain and was scaled-up successively to 50 ml, 100 ml, 250 ml, 500 ml and finally 2 L. In order to reach high efficiency in algae biomass production, scale-up is necessary to avoid nutrients from being limiting factor in the cultures. (Wolf et al., 2015)

Table 2. F/2 Medium recipe (Guillard, 1975)

F/2 Medium			
Stock	*Component*	*Stock solution (g/L or mL/L)*	*Volume per L (mL)*

I	$NaNO_3$	75 g	1
II	NaH_2PO_4 . 1 H_2O	5 g	1
III	Na_2SiO_3 . 9 H_2O	30 g	1
Trace metals	$FeCl_3$ 6 H_2O	3.15 g	1
	Na_2-EDTA . 2 H_2O	4.36 g	
	$MnCl_2$. 4 H_2O	1 mL	
	$ZnSO_4$. 7 H_2O	1 mL	
	$CoCl_2$. 6 H_2O	1 mL	
	$CuSO_4$. 5 H_2O	1 mL	
	Na_2MoO_4 . 2 H_2O	1 mL	
Vitamins	Thiamine . 1 HCl (vit B_1)	0.2 g	0.5
	Biotin (vit H)	1 mL	
	Cyanocobalamin (vit B_{12})	1 mL	

Table 3. BG11 Medium recipe (Stanier RY, Kunisawa R, Mandel M and CohenBazire G, 1971)

BG11 Medium			
Stock	*Component*	*Stock solution (g/L)*	*Volume per L (mL or g)*
I	$NaNO_3$		1.5 g
II	K_2HPO_4	40	1 mL
III	$MgSO_4$. 7 H_2O	75	1 mL
IV	$CaCl_2$. 2 H_2O	36	10 mL
V	Citric Acid . 1 H_2O	6	10 mL
VI	Ammonium Ferric Citrate	0.6	10 mL
VII	Na_2-EDTA . 2 H_2O	0.1	10 mL
VIII	Na_2CO_3	2	10 mL
Trace Metals	H_3BO_3	2.86	1 mL
	$MnCl_2$. 4 H_2O	1.81	
	$ZnSO_4$. 7 H_2O	0.22	

	$Na_2MoO_4 . 2 H_2O$	0.39
	$CuSO_4 . 5 H_2O$	0.08
	$Co(NO_3)_2 . 6 H_2O$	0.05

Table 4. BBM Medium recipe (Bischoft and Bold, 1963)

BBM Medium			
Stock	*Component*	*Stock solution (g/L or mL/L)*	*Volume per L (mL)*
I	$NaNO_3$	25.00 g	10
II	$CaCl_2 . 2 H_2O$	2.50 g	10
III	$MgSO_4 . 7 H_2O$	7.50 g	10
IV	K_2HPO_4	7.50 g	10
V	KH_2PO_4	17.50 g	10
VI	NaCl	2.50 g	10
Alkaline EDTA solution	EDTA (Titriplex III)	50.00 g	1
	KOH	31.00 g	
Acidified Iron solution	$FeSO_4 . 7 H_2O$	4.98 g	1
	H_2SO_4	1 mL	
Boron solution	H_3BO_3	11.42 g	1
Trace Metals solution	$ZnSO_4 . 7 H_2O$	8.82 g	1
	$MnCl_2 . 4 H_2O$	1.44 g	
	MoO_3	0.71 g	
	$CuSO_4 . 5 H_2O$	1.57 g	
	$Co(NO_3)_2 . 6 H_2O$	0.49 g	

Growth curves had a reserved space in the culture room and LED lights following the scheme in Figure 1 provided the light.

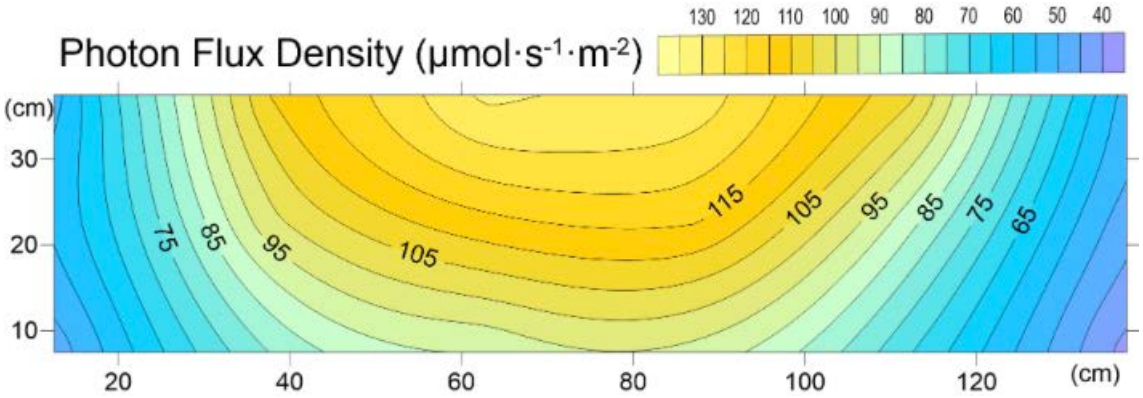


Fig.1 Light scheme of the growth curves expressed in µmol m^{-2} s^{-1}.

3.4. Experimental design

3.4.1. Calibration curves

Calibration curves of the strains BEA0069, BEA0505B, BEA1211B, BEA1267B and BEA0046 were established to get the relationship between optical density (OD) and dry weight (DW). A sample of the culture was diluted in five serial dilutions (10, 25, 50, 75 and 100%), the OD was measured in triplicate (Griffiths, 2011). Samples of 5 mL were filtered to carry out dry weight (DW) determinations. Due to the quantity of dissolved salts in the medium, ammonium formate 0,5 M was used to determine the DW (Arredondo and Voltolina, 2007).

For the calibration curve of BEA1211B the fresh weight/dry weight (FW/DW) relationship was calculated. Samples were filtered and weighted in the flow-hood to obtain FW. Then, samples were dried in the stove 24 hours at 60º C to obtained DW and made the FW/DW relationship. FW/DW relationship was calculated as a mean value of 10 different replicates.

3.4.2. Washing methods comparative

In some cases, the non-cell suspended solids like calcium carbonate, can affect the results. To rule out such effects, three calibration curve methods are tested during the experiment. The first without washing (UnW), the second by washing with distilled water (DiW) and the thirst by washing with ammonium formate (AFW) (Zhu and Lee, 1997). Cell washing reduces calcium carbonate, which can affect the results by increasing dry weight.

A sample of BEA0069 was diluted by triplicate in five serial dilutions (10, 25, 50, 75 and 100%) for each washing method. 1) UnW treatment, 5 mL of each dilution was filtered directly. 2) DiW treatment, 5 mL of each dilution were filtered and then 10 mL of distilled water was added to the filter in the flow-hood. 3) AFW treatment, 5 mL of each dilution were filtered and then 10 mL of ammonium formate (0,5 M) was added to the filter in the flow-hood.

Filters stayed 24 hours in the dissecator in order to determine DW and OD was measured with 2 mL of each dilution by triplicate.

3.4.3. Optical density (OD) determination

Optical density (OD) measurements were carried out using a Perkin Elmer Lambda 25 Spectrophotometer. Samples triplicates (1.5 mL) were measured at A680 and A750 and OD calculated as:

$$OD = A680 - A750$$

3.4.4. Growth curves

Increase in cell size and cell mass during the development of an organism is named growth. One of the objectives of growth curves was to quantify, study and plot the different phases of microalgae growth. Additionally, it was usefull parameter to determine the yield in g DW L^{-1} day^{-1}.

Growth curves had several pre-established conditions for their development. Initial innoculum was 50 mg DW L^{-1} and the growth period was eight days for eukaryotes and 10 days for cyanobacteria. Harvesting was made in the afternoon starting at 3:00 p.m.

During the experimental period, measurements for OD were taken culture volume (2L) refilled with milliQ water daily.

3.4.5. Growth rate and duplication time

For calculating the specific growth rate (μ), duplication time ($t_{1/2}$) and productivity for each strain, the following equations were used:

$$\mu\ (d^{-1}) = \frac{Ln\ (N2/N1)}{(t2 - t1)}$$

$$t_{\frac{1}{2}}(d) = \frac{Ln\ 2}{\mu}$$

$$\text{Productivity}(g\ DW\ L^{-1}d^{-1}) = \frac{(N2 - N1)}{(t2 - t1)}$$

Where:

N1= value of DW at exponential growth start(t1)

N2= value of DW at exponential growth final(t2)

3.4.6. Harvesting

Most microalgae strains were harvested by centrifugation. BEA 0069, BEA 0505B were harvested by centrifugation in 1 liter recipient at 9000 rpm during 15 minutes in two times, first with the original culture medium and subsequently after rinsing the centrifugation residue with distilled water. BEA1211B and BEA1267B were harvested by filtration through a 50 μ mesh.

3.5. Fo/Chla verification

3.5.1. Chlorophyll fluorescence

Chlorophyll fluorescence Fo was measured using AP-C AquaPen-C 100. 3 mL of algal samples were dark-adapted for 30 min prior to initial (Fo) fluorescence level measurements. To obtain the Fo value, 3 mL algal samples were sampled into the AP-C AquaPen-C 100 generating a chlorophyll fluorescence induced dynamic curve (OJIP-620) under a wavelenght of 620 nm with pulse light and active light intensity of 1800 and 50 μmol m^{-2} s^{-1}. The Fo value was obtained from the exported data of OJIP-620. (Hui Chen et al., 2017). All measurements were at least performed in triplicate.

3.5.2. Quantification of Chlorophyll *a*

Methanol was used to extract chlorophylls. 1.5 mL of culture were collected in an eppendorf, centrifuged at 3000 rpm (Hettich Mikroliter) for 10 minutes (Ritchie, 2005). The supernatant was removed and the pellet was rinsed with milli Q water. After centrifugation, the cell pellet was resuspended with 1,5 mL methanol and it was stored at 4 ºC in darkness for an hour (Hui Chen et al., 2017)

The concentration of chlorophyll a was calculated by Wellburn's equations (Wellburn, 1994).

$$Chl\ a\ (\mu g.mL^{-1}) = \left(16{,}72\ .\ A_{665{,}2}\right) - (9{,}16\ .\ A_{652{,}4})$$

3.5.3. Cell counts

Cell counts were measured (every one or two days) with a Neubauer chamber under a microscope (Leica DM2000); (http://www.celeromics.com/en/resources/docs/Articles/Cell-counting-Neubauer-chamber.pdf)

6. 4. Results and discussion

4.1. Strains selection

Criteria for the selection of strains were mainly based on growth and time for scale-up from 15 mL to 2 L. Final strains took in consideration for these experiments are shown in Table 5.

Table 5
General characteristics of selected microalgae strains and growth curve method used in the study.

BEA Code	Sp Name	Phylum	Cell Morphology	Growth curve method
BEA0069	*Halochlorella rubescens*	Chlorophyta	Unicellular	OD
BEA0505B	*Entomoneis sp*	Diatom	Unicellular	OD
BEA1211B	*Leptolyngbya sp*	Cyanobacteria	Filamentous	FW/DW relationship
BEA1267B	*Leptolyngbya sp*	Cyanobacteria	Filamentous	OD
BEA0046	*Parachlorella sp*	Chlorophyta	Unicellullar	ND

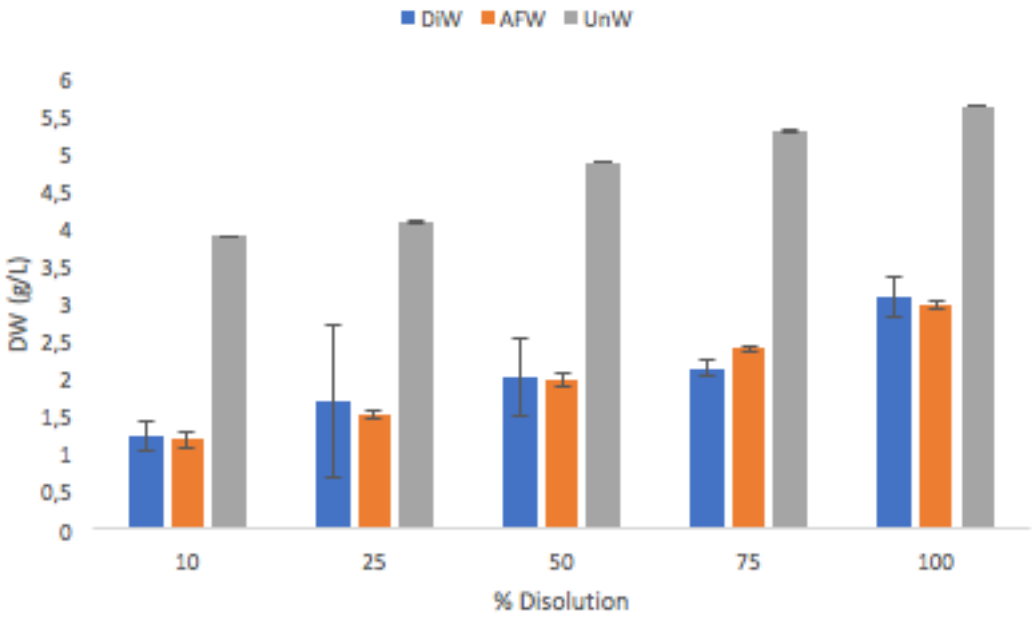
Strain selection of microalgae is important for the SABANA Project due to the projects focus on the investigation of strains with easy-growth characteristics in respect to the concept of biorefinery. Aquaculture has been rapidly expanding making it the fastest growing food production sector in the 21st century (Chopin et al., 2001). However, with increasing importance of this sector key problems have to be addressed, among these the consumption of freshwater. In this regard, the selected strains from marine sources are an interesting option and able to growth in the standard F/2 medium.

The experiments were focused on fast growth and easy-culture strains for their commercial development. In consequence, BEA0069, BEA0045, BEA1267B, BEA1211B and BEA0505B were the selected strains to characterize their growth because of their ability to reach an optimum density that allows to perform growth curves.

4.2. Calibration curves

4.2.1. Washing methods comparative

Calibration curves were a time comsuption proceeding due to the problem of salts accumulation in the filters. To solve it three methodologies were tested in BEA0069 i.e, without washing (UnW), washing with distilled water (DiW) and washing with ammonium formate 0.5 M (AFW).



Fig,2 Comparison of the differents methods used for DW calculation with BEA0069.

In BEA0069, DW of non-washed samples is at least higher by a factor of two than those washed with distilled water and 0,5 M ammonium formate (Fig.2). While those samples washed with distilled water show a high variance in DW due to osmotic shock that broke cell walls, washing with ammonium formate was established as the standard method so it was the best method to perform calibration curves while DiW is the worst method because caused an osmotic shock that led to variation of the DW.

The importance of calibration curves lies in knowing accurately the initial concentration to start growth curves.

4.2.2. Calibration curves

Calibration curves were performed for BEA0069, BEA0505B and BEA1267B (Fig.3). Unfortunately, the calibration curve of BEA0045 could not be performed. The reason for this is that BEA0045 is heavy and falls to the bottom quickly to measure in the spectrophotometer main OD measurement impossible. With calibration curves in hand, the correlation equations could be obtained where y= g DW/L and x=OD (Table 6). With this correlation equation, initial inoculum of growth curves can be calculated.

Another way to obtain BEA0045 calibration curve is to establish the relationship between Chlorophyll a and DW. However, the morphology of the cell and its thick wall did not allow for this (Krienitz et al, 2004).
The method used to obtain the calibration curve for BEA1211B was to establish the relationship between FW/DW (Table 6).

Table 6.
Correlation equations of the calibration curves for microalgae species used.

BEA Code	Corr.Equation	R^2	FW/DW relationship
BEA0069	y=0.0194x+0.9951	0.9971	-
BEA0505B	y=0.0634x-0.0225	0.9761	-
BEA1267B	y=27.236x+0,627	0.9723	-
BEA1211B	-	-	12.2220

4.3. Growth curves

Optical density (BEA0069, BEA0505B, BEA1267B) and FW/DW (BEA1211B) relationship were used to estimate the growth of each culture. The BEA0069 growth curve has four days of lag-phase and a slow transition to stationary phase with a short exponential phase (Fig.3A). For BEA0505B the exponential growth took longer and could still keep on growing after the study (Fig.3A). Exponential growth is preceded by a short lag-phase and BEA1211B (Fig.3C) showed a pattern similar to BEA0505B. BEA1276B (Fig.3A) presents problems to measure OD when it starts the growth.

Results are probably misleading due to agglomeration of cells during the lag-phase (day 2) and since then disaggregation and homogenization of the culture was impossible to obtain. For that reason, the correct method to establish BEA1267B growth curve is FW/DW relationship as in BEA1211B (Fig. 3C).

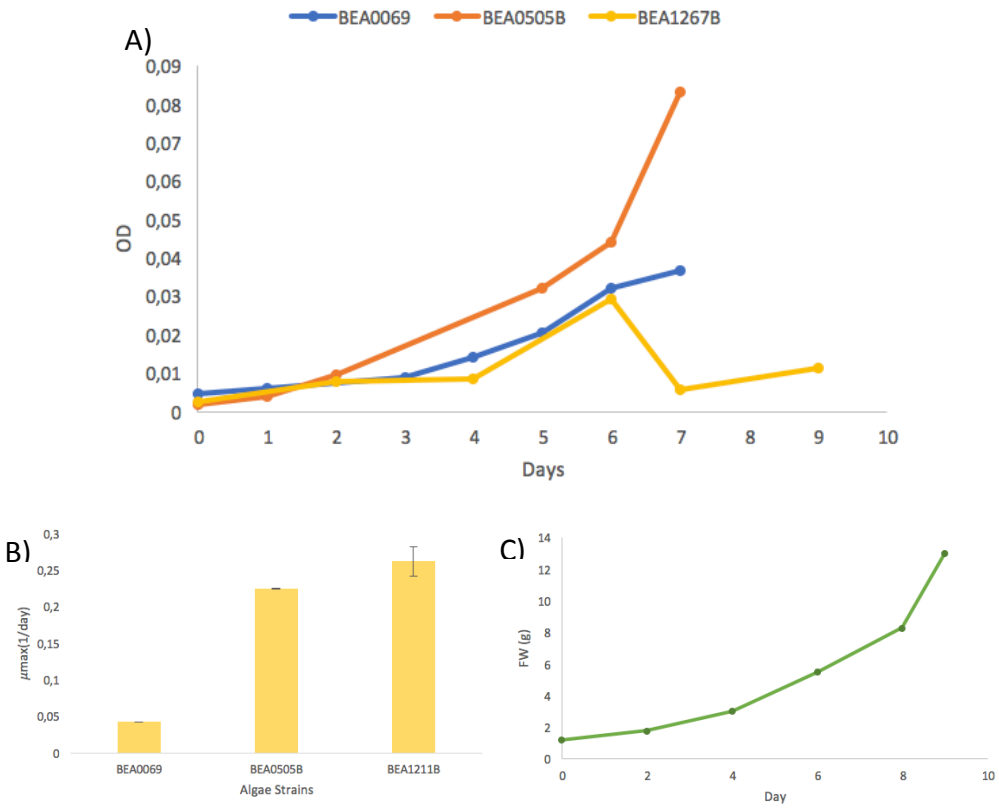


Fig.3 A) Growth curves of BEA0069, BEA0505B and BEA1267B. Growth was evaluated by OD. B) Comparison of the obtained maximum growth rates for BEA0069, BEA0505B and BEA1211B. Vertical lines represent variance of growth rates. C) Growth curve of BEA1211B. Growth was evaluated by FW.

Comparing maximum growth rates (μ), duplication time and production (Table 7) it is shown that BEA1211B had excellent growth, showing μ of 0.262 ± 0.020 d^{-1} (Fig,3B) as well as the by far the best results in duplication time and production (Table 7). Moreover, BEA1211B displays the largest stationary phase but its morphology leads to problems in the photobioreactor as it sticks to the walls and grows in cumulus. In this concern, literature has been published discussing the difficulties of making rigorous comparisons between different algae growth experiments considering variations in culture conditions (Griffith et al., 2009).

Two methods were used for harvesting, namely centrifugation for BEA0505B and BEA0069 and 50μ mesh for BEA1211B and BEA1267B.

Table 7.

Growth parameters obtained from growth curves data.

BEA Code	$\mu(d^{-1})$±SD	Duplication time (d) ±SD	Production (g DW/L d) ±SD	Biomass harvested (g FW)
BEA0069	0.042 ±0.000	10.752±2-037	0.054±0.000	31.712
BEA0505B	0.224±0.0316	3.139±0.381	0.195±0.067	27.543
BEA1211B	0.262±0.141	2.648±0.766	0.307±0.032	110.830
BEA1267B	-	-	-	24.440

SABANA Project establishes three main starting hypotheses: a) culture starts with 50 mg/L, b) duration of the curve for cyanobacteria is 10 days and for eukariotes eight days and c) the harvesting is done in the afternoon. Biological activity and productivity could vary between strains of the same species this could be due to different physiological states during the harvest. It is well known that growth conditions affect biological activity and the synthesis of secondary metabolites (Ördög et al., 2004). As a result, with the starting hypothesis achieved two things, the standard growth for the cultures and maximizing the synthesis of secondary metabolites.

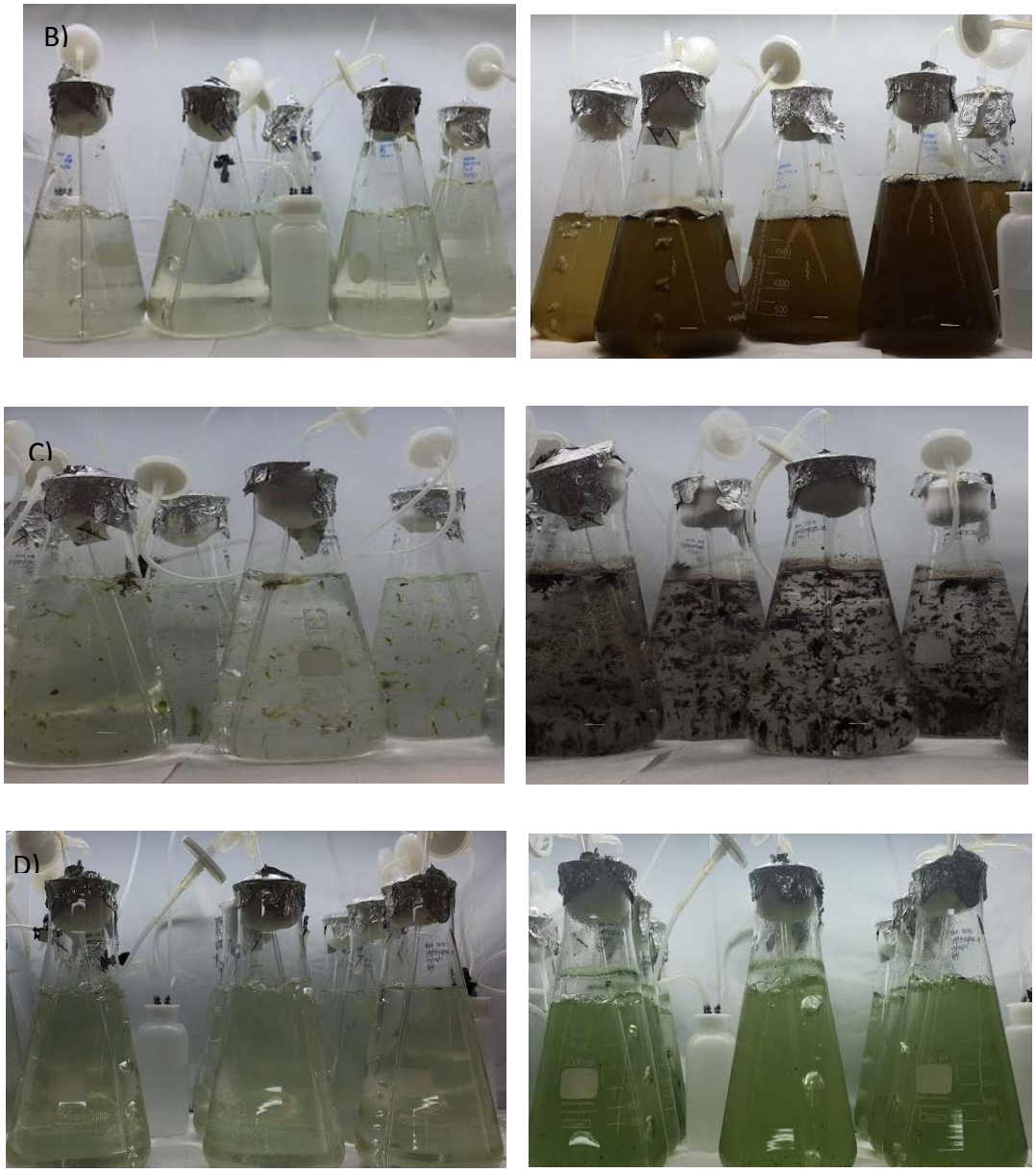


Fig.4 Evolution of the cultures over time. On the left: day zero and on the right: final day for growth curves experiments. A) BEA0069, B) BEA0505B, C) BEA1211B and D) BEA1267B.

Harvesting of strains was not homogeneus in all the replicates and diferences in biomass content between the erlenmeyers with more light intensity and the other ones with less light intensity could be observed. SABANA Project estimated to reach at least 8 g DW in each growth curve but only in BEA1211B it seemed that the estimated amount was reached. For future essays, several modifications are already envisioned. First, cultures should be bubbled with air +1.5% CO2 in order to have a faster growth (Ördög et al., 2004), second the growth curves should be carried out with more replicates, at least 15 erlenmeyers to reach the necessary 8 g DW.

4.4. Fo correlation with Chla

BEA0505B, BEA1267B and BEA1211B were selected to investigate the relationship between Fo and Chla. Fo is a basal chlorophyll fluorescence parameter, which is the fluorescence yield of PS II reactions centers opened completely (Chen et al., 2017). Fo and Chla were measured during the performance of growth with different microalgae morphology, i.e unicellular (BEA0505B), filamentous (BEA1267B and BEA1211B). Correlation equations were established to confirm the relationship between Fo and Chla (Fig 5). Once the Chl a/Fo relationship is verified it could be useful to establish a method to estimate primary productivity by measuring Fo value (Chen et al., 2017). Although all of the tested strains obtained a good linear relationship BEA0505B had got the best results.

Fo and Chl a showed significant linear relationship in all of the microalgae regardless of their phylum and morphology. Chlorophyll extraction is rather time consuming while the fluorescence method is simple and easy to operate. Fo is a basal chlorophyll fluorescence parameter and Chl a is one of the basic parameters to estimate primary production and a good indicator of biomass (Chen et al., 2017). Finally, with the established Fo/Chl a relationship the Chl a content could be determinated by only measuring Fo content and subsequently calculating the primary production.

A) B)

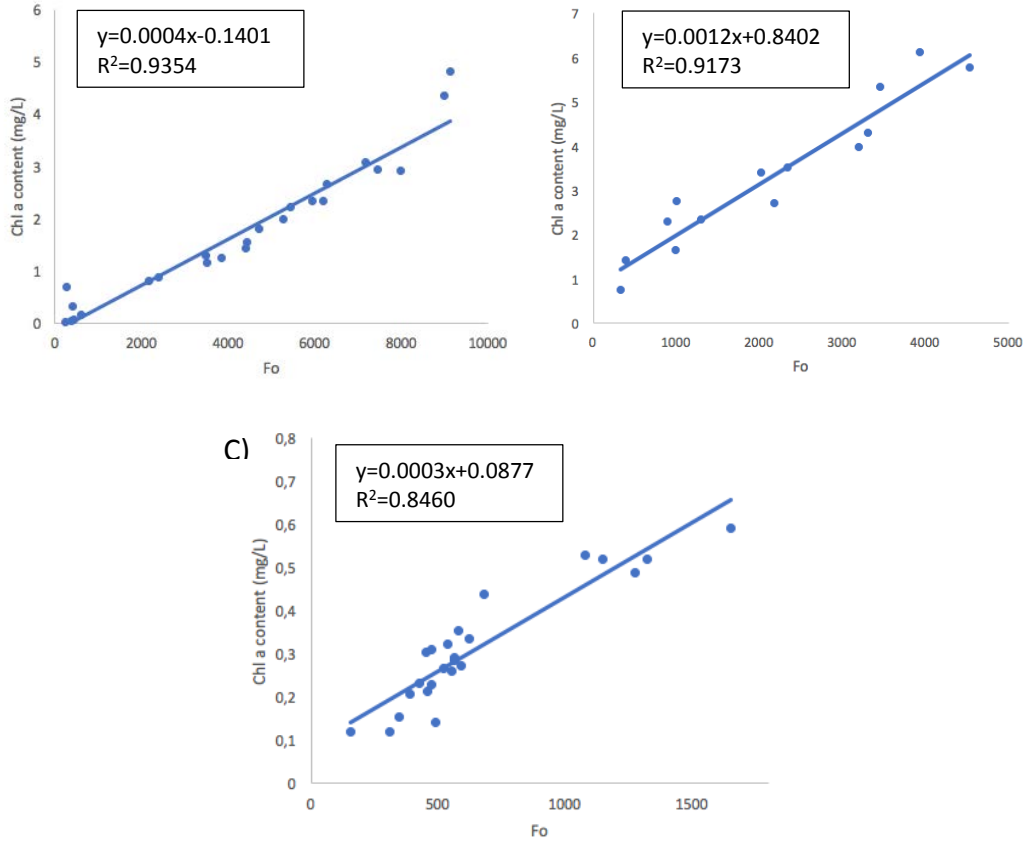


Fig.5. Fo-Chl a content correlation curves of the three microalgae used. A) BEA0505B, B) BEA1211B and C) BEA1267B.

7.

8. 5. Conclusions

(1) The experiments started with the culture of 18 microalgae strains under controlled conditions. BEA0069 (*Halochlorella rubescens*), BEA0505B (*Entomoneis sp*), BEA1211B (*Leptolyngbya sp*), BEA1267B (*Leptolyngbya sp*) and BEA0045 (*Parachlorella sp*) were the microalgae that showed the fastest growth rates. Of these strains only BEA0045 could be used to continue with the essays because the OD and Chl a extraction was not compatible with the methodology used.

(2) The best method to investigate calibration curves was found out to be the washing with ammonium formate 0,5M instead of no washing or washing with distilled water. The ammonium formate solution was able to reduce the salt in cells (by measuring weight) by an order of magnitude.

(3) Growth curve perfomance served to characterize microalgae growth using two methods. The first followed growth from the OD (BEA0069, BEA0505B, BEA1267B) , the second used the the FW/DW relationship (BEA1211B). The strain BEA1267B could not be used to performed growth curve due to incompatibility of the envisioned method. It should be kept in mind for further investigation that the FW/DW method should be used instead of OD.

(4) The highest growth rates (0,262 d^{-1}), production (0.307 g DW L^{-1} d^{-1}) and the shortest duplication time (2.648 d) were found for strain BEA1211B.

(5) The Fo/Chl a linear relationship was verified for all of the strains using Fo as a simple, rapid and inexpensive measure showing that it is a useful method to estimate productivity related to Chl a content.

9. 6. Bibliography

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10. 7. Webgraphy

http://www.algaebase.org

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http://www.eu-sabana.eu

http://www.fao.org/docrep/005/x3980e/x3980e07.htm

http://www.marinebiotechnology.org/es/

Descripción de las actividades realizadas durante la realización del TFT

Desde un principio he desarrollado mis prácticas dentro del proyecto SABANA (Sustainable Algae Biorefinery for Agriculture aNd Aquaculture), este proyecto se desarrolla a nivel europeo junto con otras 13 instituciones colaboradoras. A modo de resumen, el objetivo del proyecto es la producción de biopesticidas y bioproductos que sean estimulantes de la actividad agrícola y la acuicultura. Estas sustancias se obtendrán de microalgas y cianobacterias de una forma sostenible, utilizando el agua del mar y aprovechando los recursos disponibles en aguas residuales.

El papel del BEA es la de hacer una selección de cepas de agua marina que puedan tener una aplicación en estos tres casos.

Mi función ha sido la de iniciar el crecimiento de los cultivos a ensayar e ir escalando los cultivos para poder, en última estancia estudiar el crecimiento de estos mediante la realización de curvas de crecimiento y por último cosecharlos.

Otra de mis funciones ha sido la verificación en el laboratorio de una técnica en el que, a través de medidas de la fluorescencia de la clorofila, se obyienen valores de producción a partir de las relaciones que se establecen entre la F_0 (fluorescencia basal)

y otras medidas de biomasa (clorofila a, peso seco) o densidad celular.

Formación recibida

Durante mi estancia en el BEA, desde el 27 de enero hasta el 12 de mayo he tenido la oportunidad de desarrollar una serie de tareas que no hubiesen sido posibles sin los conocimientos adquiridos en las primeras semanas de prácticas externas. Durante este tiempo siempre he tenido la ayuda de mis tutores dentro del centro que han estado dispuestos siempre que ha sido necesario.

Nivel de integración en el centro

Desde el primer momento me he sentido uno más del personal del BEA, el ambiente de trabajo ha sido bueno y todo lo que he podido aprender ha sido gracias a ellos y a mis compañeros de prácticas.

Aspectos positivos y negativos

Ha sido mi primera experiencia “profesional” y ha sido muy positiva porque tengo la sensación de que mis cuatro años en los que he cursado el grado en Ciencias del Mar han tenido resultado y he podido aplicar algunos de mis conocimientos. He tenido la oportunidad de aprender y siento que he adquirido muchos conocimientos y práctica que me podrán servir para un futuro. El equipo del BEA me ha tratado muy bien en todo momento, en especial mis dos tutores, Mireia Sánchez (en el laboratorio) y Juan Luis Gómez Pinchetti.

Un aspecto negativo ha sido que el trabajo ha requerido tiempo de estar en el laboratorio y me ha dejado poco tiempo para escribir el TFT. Me hubiese gustado obtener los resultados un poco antes y poder escribir con más tranquilidad.

Valoración personal

Ha sido una experiencia muy positiva y gratificante en todos los sentidos, el personal del BEA me ha tratado como uno más, mis compañeros de prácticas han sido

muy buenos y muy capacitados para hacer cualquier tarea, también he aprendido mucho de ellos. Como ya he mencionado he adquirido muchos conocimientos y me siento capacitado para poder desarrollar mi trabajo en otros lugares.